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IRON-LIMITED GROWTH KINETICS OF THIOBACILLUS FERROOXIDANS ISOLATED FROM ARSENIC MINE DRAINAGE

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IRON-LIMITED GROWTH KINETICS OF *THIOBACILLUS FERROOXIDANS* ISOLATED FROM ARSENIC MINE DRAINAGE

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IRON-LIMITED GROWTH KINETICS OF THIOBACILLUS FERROOXIDANS ISOLATED FROM ARSENIC MINE DRAINAGE

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ABSTRACT

Thiobacillus ferrooxidans is found in heavy-metal contaminated drainages from placer and lode gold mines in many parts of Alaska and Canada. I have examined the iron-limited growth kinetics of an T. ferrooxidans isolate, AK1, using continuous cultures. AK1 is an arsenic tolerant isolate obtained from placer gold mine drainage containing large amounts of dissolved arsenic.

The steady state growth kinetics can be described by a simple Monod equation modified for threshold ferrous iron concentrations. A supplement of 200 $mg \cdot l^{-1}$ reduced arsenic to the ferrous medium did not result in an increased steady state biomass nor did it appear to affect the steady state growth kinetics obtained in continuous cultures.

Kinetic descriptions of the iron-limited growth of acidophilic iron-oxidizing bacteria are important for understanding the biogeochemical cycling of many metals and for application of these microorganisms to biohydrometallurgy.

TABLE OF CONTENTS

LIST OF FIGURES

Page

Page

LIST OF TABLES

PREFACE

"It is probably not unscientific to suggest that somewhere or other some organism exists which can, under suitable conditions, oxidise any substance which is theoretically capable of being oxidised."

E.F. Gale, 1952

It is impossible to thank all of the people who have supported me throughout my graduate studies. I would, however, like to acknowledge and thank the entire staff and students of IWR for their patience and advice. I would also like to thank the Department of Microbiology and Public Health at Michigan State University for their hospitality.

I would particularly like to thank: my committee chairman, Ed Brown, for his encouragement and readily available ear; Huan Luong for his knack of coming up with the strangest ideas that seem to work; and to Alan Braley for his wizardry with computer graphics.

Additionally, I would like to thank the other members of my committee: Carol Feist, whose enthusiasm for microbiology was contageous; and Dan Hawkins for his geochemical perspective in a sea of biologists.

INTRODUCTION

Arsenic contamination of groundwaters and streams occurs in the vicinity of gold deposits and placer mines in many parts of Alaska and northern Canada (Smecht et al., 1975; Wilson and Hawkins, 1978; Brooks et al., 1982; Brown et al., 1982). A recent revitalization of mining and mineral exploration (see Edmondson, 1980) in these geographical areas has created a need for research designed to gain a better understanding of biogeochemical processes related to mining activities. This type of research can help answer problems of environmental concern and can potentially aid in the development of economically feasible methods of successfully mining lower grade ores.

Thiobacillus ferrooxidans is the bacterium which is responsible for heavy metal leaching and acid production in water draining from sulfide deposits. T. ferrooxidans can catalyze the dissolution of heavy metals either by directly attacking sulfide ores or by oxidizing reduced iron leading to indirect dissolution via the ferric ion (Brierley, 1982). Since sulfides and heavy metals such as arsenic are commonly associated with lode-gold deposits and are sometimes associated with placer deposits (Wilson and Hawkins, 1978), we reasoned that T. ferrooxidans may play a role in the heavy-metal contamination of streams and groundwaters that are impacted by mining activities.

Brown et al. (1982) found large numbers of T. ferrooxidans in drainage from 90% of the placer mines that they sampled. Greater than 10 parts per billion (ppb) of the dissolved metal arsenic were found in 30% of those same streams. In addition, T. ferrooxidans and arsenic were present in streams affected by lode mining (Forshaug et al., 1982). These results suggest that T. ferrooxidans may be directly involved in the leaching of arsenic and other heavy metals associated with active and abandoned mines in subarctic streams. However, the mechanisms by which these organisms are involved in the cycling of heavy metals, such as arsenic, are not fully understood.

Many investigators have studied the microbiologically mediated dissolution kinetics of various sulfide minerals (Torma and Habashi, 1972; Silver and Torma, 1974; Karaivko et al., 1977; Lundgren and Silver, 1980; Hoffman et al., 1981; Chang and Myerson, 1982). In addition, the growth kinetics of various Thiobacillus species when grown on reduced sulfur compounds have been described (Bounds and Colmer, 1972; Eccleston and Kelly, 1978; Justin and Kelly, 1978; Leefeldt and Matin, 1980). However, the iron-limited growth kinetics of Thiobacillus ferrooxidans are not well understood. The partial kinetic descriptions that are found in the literature (Lacey and Lawson, 1970; MacDonald and Clark, 1970; Tomizuka et al., 1976; Guay et al., 1977; Kelly et al., 1977; Kelly and Jones, 1978) show inconsistencies in results. This may be due to the difficulties of growing this organism, particularly in continuous culture and to the

taxonomic uncertainty of isolated strains of acidophilic iron oxidizers (DiSpirito et al., 1982).

In this study I will describe the iron-limited growth kinetics, using both batch and continuous culture data, of a T. ferrooxidans strain isolated from a Fairbanks, Alaska area stream which is impacted by placer mining and which contains large amounts of dissolved arsenic. In addition, I will describe the potential of this organism to use reduced arsenic as an energy source for growth. These types of studies should help elucidate the mechanisms contributing to the leaching of heavy metals such as arsenic into streams and groundwaters. An understanding of the mechanisms involved in heavy metal leaching will be useful for hydrometallurgical applications and for the development of adequate, rational long-range solutions to potential water problems caused by or related to placer and lode-gold mining.

LITERATURE REVIEW

Microbial Leaching of Metal Sulfides

The presence of metals such as copper and uranium from leach waters of mine drainage was observed historically by the Phoenicians, Romans, Arabs, and Spaniards (Torma, 1976; Kelly et al., 1979; Brierley, 1982). However, the contribution of microorganisms in these processes was not realized until the 1920s. It was not until 1947 that the microorganism responsible for these leaching processes, Thiobacillus ferrooxidans, was isolated from drainage from bituminous coal mines (Torma, 1976). The significance of the growth parameters of these organisms (very high tolerance for heavy metals and acidity) has only recently been realized.

Considerable economical and environmental interest has been shown in these microorganisms. Economically, their leaching capabilities may be used to recover metals from low-grade sulfide ores. In the U.S. leaching of low-grade copper waste materials accounts for about 11.5% of the total annual copper production (Brierley, 1978; Kelly et al., 1979). As high grade ores become depleted, the bacterial enhancement of metal leaching should become increasingly important. Environmental interest has been centered around the role of these organisms with respect to catalyzing chemical reactions leading to acid mine drainage and to the leaching of toxic heavy metals. Additionally, the ability of these organisms

to oxidize sulfur compounds may be utilized in the near future as an economically feasible method for the commercial desulfurization of coal (Hoffman et al., 1981).

The mechanisms by which bacteria leach metals from sulfide minerals have been examined by many investigators (Silverman and Ehrlich, 1964; Silverman, 1967; Duncan and Drummond; 1973; Gaidarjiev et al., 1975; Bennett and Tributsch, 1978; Groudev, 1979). By convention, the mechanisms have been divided into two types: direct and indirect bacterial leaching (Brierley, 1982).

The mechanism of direct bacterial leaching was first proposed by Silverman and Ehrlich (1964). This mechanism requires physical contact between the bacteria and the mineral surface and involves an enzymatic attack by the bacteria on components of the mineral which are susceptible to oxidation (Silverman, 1967; Brierley, 1982). The bacteria gain energy for growth by a transfer of electrons from the reduced component (i.e. sulfur, iron, etc.) to oxygen. Oxidized products, which are generally more soluble than the reduced species, are then released (Brierley, 1982).

Several types of evidence suggest that direct leaching by the thiobacilli is possible. First, it has been observed that bacteria accelerate the rate of oxidation of iron-free synthetic metal sulfides (Silverman, 1967). Second, scanning electron microscopy has been used to observe mineral surfaces before and after exposure to leaching. Duncan and Drummond (1973) and Bennett and Tributsch (1978) found that pyrite crystals which had been exposed to leaching

were deeply etched in characteristic patterns. Both authors concluded that these observation support the direct-attack theory. Last, Gaidarjiev et al. (1975) used heterotrophic strains and ultra-violet (UV) light mutant autotrophic strains of thiobacilli, neither of which had ferro-oxidase activity, and compared kinetic leaching data to that of wild-type autotrophic strains. They concluded that the heterotrophic and mutant strains which were unable to produce ferric ion were able to directly attack sulfide minerals.

Indirect leaching involves the bacterial oxidation of soluble ferrous iron to ferric iron. Ferric iron, a powerful oxidizing agent, can then react with various reduced metals present in the mineral complex transforming them into the more soluble oxidized forms. This mechanism has also been referred to as bacterially assisted leaching (Brierley, 1982).

Singer and Stumm (1970) found that the rate limiting step in indirect leaching is the regeneration of ferric iron. They also showed that, in the absence of bacteria, ferrous iron is stable in acid solution and, therefore, leaching by ferric iron occurs very slowly. However, when T. ferrooxidans was present the oxidation of ferrous to ferric iron was accelerated by a factor of greater than 10^6.

Although there has historically been some controversy over which mechanism of bacterial leaching is most important, it is now fairly well accepted that both mechanisms contribute to the

dissolution of metals in the environment. Since most minerals contain some iron, both mechanisms may exist concurrently or in succession (Lundgren and Silver, 1980; Brierley, 1982).

The rate of metal dissolution from metal sulfides is dependent on environmental factors which influence bacterial growth such as temperature, pH, and availability of nutrients (Tuovinen and Kelly, 1972). Torma and Sakaguchi (1978) also found that the rate of oxidation by T. ferrooxidans was directly proportional to the initial surface area of the substrate and most importantly was related to the solubility product of the substrate. The highest rates of dissolution were obtained with the substrate having the highest solubility product.

Since the original isolation of these organisms, it has become increasingly apparent that they are involved in many biogoechemical processes involving the dissolution of sulfides. Because of the ubiquitous nature of the organisms in any environment containing sulfides and acidity, many biogeochemical roles have recently been described for these organisms. As more is known about these microorganisms it is increasingly apparent that many more roles will be discovered.

Arsenic and Thiobacillus ferrooxidans

In Alaska and many parts of Canada, large amounts of arsenic-containing minerals are often closely associated with

sulfides and gold-bearing minerals (Smecht et al., 1975; Wilson and Hawkins, 1978). The mobilization of arsenic appears to be a result of exposure of these arsenic-containing minerals to natural weathering processes. The conditions which exist in Fairbanks area streams should thermodynamically favor immobilization of the more toxic arsenite species to arsenate by oxidation and precipitation reactions leading to the formation of minerals such as scorodite (Wilson and Hawkins, 1978). However, the rates of these reactions are not completely defined nor are the effects of greater than normal exposure of arsenic-containing rocks to massive disturbances such as from mining activities. Since placer, suction dredge and lode-gold mining activities have recently increased in Alaska, many streams are now affected by mining.

The occurrence of dissolved arsenic in the Fairbanks area was first reported by Wilson and Hawkins (1978). They sampled streams, stream sediments and groundwaters near Fairbanks and found arsenic concentrations in excess of the Environmental Protection Agency's recommended limit of 50 parts per billion (ppb). More recently, Brown et al. (1982) found both dissolved arsenic and T. ferrooxidans in streams in subarctic Alaska and Canada which are impacted by placer gold mining.

The streams sampled by Brown et al. (1982) were all neutral in pH. T. ferrooxidans is normally found in acid streams draining from mines that have pyrite deposits. However, these bacteria have also recently been found in neutral and alkaline waters affected by coal

strip mining in southeastern Montana (Olson et al., 1979). Brown et al. (1982) suggest that T. ferrooxidans are not actively growing in these streams but rather, as part of the mining process, large populations of these bacteria are being washed down with particulate matter which may contain heavy metals such as arsenic. Since T. ferrooxidans is obligately acidophilic (when grown on reduced sulfur and iron compounds) it could be inferred that "hot spots" must exist where the environmental conditions favor the growth of this organism.

Very recently, Forshaug et al. (1982) found waters associated with two abandoned lode mines which were highly acidic (pH 2.2-2.6), had greater than 10^6 T. ferrooxidans per milliliter and contained large amounts of dissolved arsenic. Small pools near a holding pond at the first mine contained greater than 50 ppb of dissolved arsenic. Arsenic was present despite the fact that arsenic-containing minerals were not detected in tailings which were associated with the pools. At the second mine, a stream flowing through abandoned tailing piles was found to have dissolved arsenic concentrations of as high as 19 parts per million (ppm). These recent studies suggest that T. ferrooxidans may be directly involved in the leaching into subarctic streams of arsenic and other heavy metals associated with active and abandoned mines.

In laboratory studies Ehrlich (1964) demonstrated that T. ferrooxidans can accelerate the oxidation rate of arsenopyrite several times over that of abiotic controls. This oxidation leads

to extensive solubilization of arsenic as arsenite and arsenate species. Using pyritic materials from the Fairbanks area, Luong et al. (1981) showed that the rate of arsenic leaching in the presence of T. ferrooxidans was far greater than in its absence. These laboratory experiments demonstrate that T. ferrooxidans can catalyze the solubilization of arsenic from arsenic-containing minerals.

Both the ecological control of acidity and toxic metal solubilization by T. ferrooxidans and the economic potentials in using microorganisms to recover precious metals from low-grade ores require a better understanding of the factors influencing the growth of these microorganisms.

Iron-limited Growth Kinetics

The regeneration of an oxidant (ferric iron, in the dissolution of heavy metals from iron-containing sulfide ores) is essential in the maintenance of the leaching cycle. Bacteria, particularly T. ferrooxidans, are known to accelerate the rate of iron oxidation under leaching conditions by a factor of greater than 10^6 (Singer and Stumm, 1970). Despite this fact, very little work has been done to describe the iron-limited growth kinetics of T. ferrooxidans.

Some kinetic data have been collected using batch culture techniques but very little have been collected using continuous culture techniques. This lack of data appears to be related to the difficulties of growing T. ferrooxidans with ferrous iron as a sole

energy source, particularly in continuous culture. One of the most plaguing problems has been the selection of optimum conditions to assure precipitate free systems and to avoid bacterial growth on the culture vessel walls (D.P. Kelly, personal communication). Steady-state growth kinetics such as those of Monod (1942) are of little value under non-steady state conditions such as those which would occur as a result of precipitation or bacterial wall growth.

A summary of kinetic data for iron-grown strains of T. ferrooxidans was compiled and is found in Table 1. Several difficulties exist in comparing the data. These include differences in culturing conditions (i.e. pH, temperature, initial iron concentration), in the type of system utilized (batch culture or one or two phase continuous culture), as well as potential differences in various strains which have been classified as T. ferrooxidans (see Taxonomy section).

Although the iron-limited kinetic growth data for T. ferrooxidans is inconsistent (see Table 1), most authors have determined that the maximum growth rate (μ_{max}) is about 0.1-0.2 hr^{-1}. Even less consistency exists in the values for the growth half saturation constant (K_{μ}). Most of the published values for K_{μ} are about 10-40 mM, however, several authors calculated values which are about an order of magnitude smaller.

Most of the data presented in Table 1 were collected by authors who were interested in the optimization of growing conditions for the use of this organism in the recovery of valuable metals. Thus,

Table 1. Growth kinetics of Thiobacillus ferrooxidans.

μ_{max} (hr^{-1})	K_{μ} (mM)	Temp (°C)	pH	Reference
0.143	36	30	1.6	[1]Kelly and Jones,1978
1.25-1.78	0.7-2.4	30	1.6	[2]Jones and Kelly,1977 [2]Kelly et al.,1977 [2]Kelly and Jones,1978
0.12	18-36	20	2.2	[1]Lacey and Lawson,1970
0.20	18-36	31	2.2	[1]Lacey and Lawson,1970
0.22	--	--	--	[2]Hoffman et al.,1981
0.14	37	30	2.7	[2]Tomizuka et al.,1976
0.145	7.2	32	1.8-2.0	[1]MacDonald and Clark, 1970
0.161	3.8	32	2.2	[2]MacDonald and Clark, 1970

[1] Data collected from batch culture experiments.
[2] Data collected from continuous culture experiments.

many of the kinetic values which are reported are not based on exhaustive kinetic experimentation. In addition, at least one publication (MacDonald and Clark, 1970) mentioned that deposits of bacteria and iron on the sides of the culture vessel were a problem.

At least one continuous culture kinetic study has been completed as a Ph.D. thesis (Jones, 1974), and this work has been referenced in abstracts and proceedings (Jones and Kelly, 1977; Kelly et al., 1977; and Kelly and Jones, 1978) but the work itself has not been published (D.P. Kelly, personal communication). The work of Jones indicates that μ_{max} is about one order of magnitude higher; and K_{μ} about one order of magnitude lower than those reported in batch culture (Kelly and Jones, 1978) or most other continuous culture studies (see Table 1). In addition, Jones (1974) reports a complex ferric ion inhibition of ferrous oxidation which has not been observed by other workers using different strains classified as T. ferrooxidans. Only Hoffman et al. (1981), mention a slight inhibition of iron oxidation rates if soluble ferric iron was added to a batch culture at the beginning of an experiment.

It is surprising that more continuous culture kinetic data of the iron-limited growth of T. ferrooxidans are not available since there is increasing interest in biogeochemical processes catalyzed by this microorganism. The difficulties of culturing T. ferrooxidans in iron-limited continuous cultures and of growing the

organism on solid media may account for the lack of accurate kinetic data.

General Physiology

T. ferrooxidans is classified as a Gram negative chemoautotroph. Inorganic compounds serve as sole energy sources and cellular carbon is obtained by carbon dioxide fixation. *T. ferrooxidans* is a strict aerobe, requiring oxygen as a final electron acceptor in metabolism.

Although the iron-limited growth kinetics of *T. ferrooxidans* have not been adequately described, the physiological factors affecting the growth of this bacteria have been well-defined. Much of this research has been a result of the desire to optimize conditions for the bioleaching of economically valuable metals. The results of many of these studies have been compiled in recent review articles (Tuovinen and Kelly, 1972; Torma, 1976; Brierley, 1978; Lundgren and Silver, 1980). The major growth parameters include temperature, light, pH, and carbon dioxide, oxygen and nutrient (i.e. sulfate, phosphate, nitrogen, potassium, magnesium, calcium, and some trace elements) availability.

Physical parameters which have been found to affect the growth of *T. ferrooxidans* include temperature and light. The optimum temperature has been found to be in the range of 25-45°C (Torma, 1976). In one study, LeRoux and Marshall (1977) found the

interesting result that both visible and ultra-violet light inhibited the growth of some strains of T. ferrooxidans and other members of the thiobacilli.

T. ferrooxidans is an acidophilic organism, requiring a large concentration of hydrogen ions for the oxidation of ferrous iron (Apel and Dugan, 1978). Optimum initial pH values appear to be between 2.0 and 3.5 (Tuovinen and Kelly, 1974). Since T. ferrooxidans is a strict aerobe, the oxidation-reduction potential (E_h) must be maintained at a positive level. E_h values of +190 to +550 mV have been observed in culture media during ferrous iron oxidation by T. ferrooxidans (Tuovinen and Kelly, 1974).

Oxygen and carbon dioxide concentrations have been shown to affect leaching rates (Lundgren and Silver, 1980). Although little information is available as to optimum concentrations, it has been observed that increased aeration by shaking or bubbling compressed air increases the rate of iron oxidation (Tuovinen and Kelly, 1974). However, Silverman and Rogoff (1961) observed that reversible alterations of cellular morphology may occur as a result of "excessively vigorous shaking".

Nutrients such as sulfate, phosphate, nitrogen, potassium, magnesium and calcium are required in laboratory media for optimum growth of T. ferrooxidans. Although it has been observed by many investigators that sulfate is required for iron oxidation, the role of sulfate in the mechanism of iron oxidation is still not entirely clear (Ingledew, 1982). Other trace nutrient requirements are

generally supplied by impurities in the media constituents (Lundgren and Silver, 1980). Nutrient requirements are generally supplied in popular media recipes used to culture T. ferrooxidans such as the 9K medium of Silverman and Lundgren (1959). Although ammonia is generally supplied as a nitrogen source, some T. ferrooxidans strains have recently been shown to be able to assimilate nitrate for growth when reduced iron is supplied as an energy source (Tuovinen et al., 1979).

T. ferrooxidans is known to be tolerant to many metals. Tuovinen et al. (1971) found that, during iron oxidation, strains of T. ferrooxidans were able to tolerate high concentrations of zinc, nickel, copper, cobalt, manganese and aluminum. Other metals such as silver, tellurium, arsenic, molybdenum and selenium showed varying degrees of toxicity to the strains tested. Other authors have found strains of T. ferrooxidans which are unusually resistant to copper (Groudeva et al., 1981) and to silver (Sugio et al., 1981d). Luong and Brown (1981) isolated a strain of T. ferrooxidans from an arsenic-containing stream near Fairbanks, Alaska. This isolate was tolerant to greater than 900 parts per million of arsenate and arsenite, respectively, when ferrous iron was supplied as an energy source.

Because of the increasing interest in bioleaching of valuable metals, the general physiology and optimum growth parameters of T. ferrooxidans have been fairly extensively documented in the past 20 years. The observation that this chemoautotrophic organism can

thrive at the expense of ferrous iron when supplied with oxygen, carbon dioxide and a few basic nutrients has resulted in a great deal of recent speculation on the biochemical mechanisms which are involved in energy generation during iron metabolism.

Iron Metabolism

In any discussion of metabolism several factors must be kept in mind. First, how is cellular carbon incorporated; second, how is energy generated; and third, how is reducing power balanced? The mechanisms by which the chemoautotroph, T. ferrooxidans, is able to perform and integrate these functions has been of interest to many investigators. The iron-oxidizing system appears to be constitutive and independent of the sulfur-oxidizing system (Ehrlich, 1981). The following discussion will describe the most current thoughts on the metabolism of T. ferrooxidans when grown with ferrous iron as an energy source. The mechanisms presented for the growth of this organism on reduced iron can serve as a model for the potential use of other reduced metal substrates (i.e. copper, uranium, arsenic, etc.).

Carbon Dioxide Fixation: T. ferrooxidans grows under strict autotrophic conditions, obtaining all of the carbon required for biosynthesis from carbon dioxide (Tuovinen and Kelly, 1972). Fixation of carbon dioxide appears to proceed primarily by the reductive pentose phosphate cycle or Calvin cycle (Lundgren and

Silver, 1980). In addition, a small amount of carbon dioxide may be fixed by the secondary carboxylation of phosphoenolpyruvate (PEP) derived from the Calvin cycle (Tuovinen and Kelly, 1972; Ehrlich, 1981). The later system is required for the formation of some amino acids (Ehrlich, 1981).

The Calvin cycle may be expressed in the general equation (Gottschalk, 1979):

$$3CO_2 + 9ATP + 6NADH_2 \longrightarrow 1GAP + 9ADP + 8P_i + 6NAD$$

Thus, for every three moles of carbon dioxide (CO_2) fixed as glyceraldehyde-3-phosphate (GAP), nine moles of adenosine triphosphate (ATP) and six moles of reduced nicotinamide adenine dinucleotide ($NADH_2$) are required. The additional products include adenosine diphosphate (ADP), inorganic phosphate (P_i) and oxidized nicotinamide adenine dinucleotide (NAD). Energy and reducing power for CO_2 fixation are supplied by the oxidation of reduced inorganic compounds (i.e. ferrous iron). The key enzymes in the Calvin cycle are phosphoribulokinase and ribulose 1,5-biphosphate carboxylase (Gottschalk, 1979). These enzymes have been isolated from T. ferrooxidans cells (Tuovinen and Kelly, 1972).

Secondary fixation of carbon dioxide may proceed by the action of phosphoenolpyruvate carboxylase (Ehrlich, 1981):

$$\text{GAP (from the Calvin cycle)} \xrightarrow{\text{phosphoglyceromutase}} \text{2-phosphoglycerate}$$

$$\text{2-Phosphoglycerate} \xrightarrow{\text{enolase}} \text{phosphoenolpyruvate (PEP)}$$

Phosphoenolpyruvate (PEP) is then combined with CO_2:

$$PEP + CO_2 \xrightarrow{\text{PEP carboxylase}} \text{oxalacetate} + P_i$$

Regulation of CO_2 fixation in T. ferrooxidans is not well understood (Tuovinen and Kelly, 1972). The activity of phosphoribulokinase is apparently subject to competitive inhibition by adenosine monophosphate (AMP). This observed inhibition led Tuovinen and Kelly (1972) to suggest that the fixation of CO_2 in whole cells may be regulated by the energy charge of the cell.

Generation of ATP by Ferrous Iron Oxidation: The oxidation of reduced iron (Fe^{2+}, generally supplied in pure culture as $FeSO_4$) can supply the sole energy source for the growth of T. ferrooxidans. A recent review by Lundgren and Silver (1980) summarizes the physiological events by which energy is derived:

1. Ferrous iron is oxidized with the release of electrons:

$$2Fe^{2+} \longrightarrow 2Fe^{3+} + 2e^-$$

2. The reducing potential from the above reaction is then coupled to the reduction of molecular oxygen:

$$\tfrac{1}{2}O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O$$

The pair of electrons transferred yields enough energy for the formation of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) by oxidative phosphorylation. Several authors (Ingledew et al., 1977; Apel et al., 1980; Ehrlich, 1981) have incorporated the recent mechanism for the formation of ATP as presented by Peter Mitchell in the chemiosmotic theory (see Hinkle and McCarty, 1978) with iron oxidation in T. ferrooxidans. This is summarized in Figure 1.

According to chemiosmotic theory, a proton motive force (pmf)

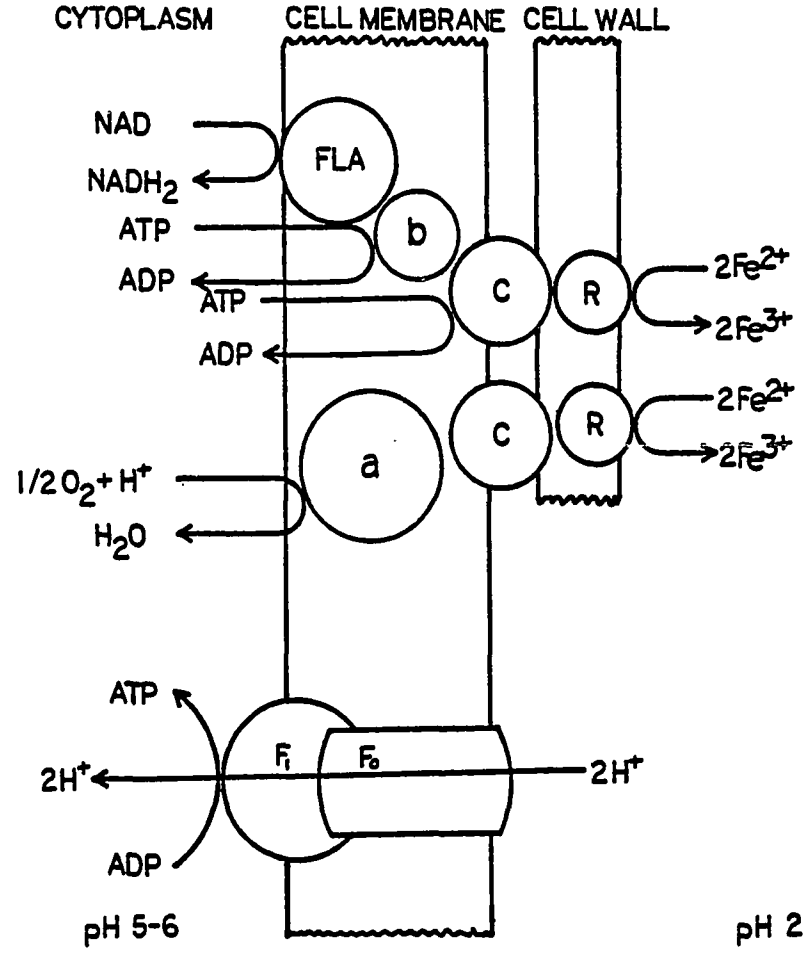

Figure 1. The proposed mechanism for energy (ATP) and reduced pyridine nucleotide ($NADH_2$) generation by T. ferrooxidans. This schematic diagram suggests the functional relationship between the cytochromes (a,b,c), an unidentified flavin (FLA) and rusticyanin (R). ATP formation proceeds by transport of hydrogen ions (H^+) via ATPase (shown here as the two subcomponents, F_1 and F_0; After Ingledew et al., 1977; Hinkle and McCarty, 1978; Ingledew and Cobley, 1980; Ehrlich, 1981; Ingledew, 1982).

can be generated from a pH gradient (ΔpH), or a difference in electrical potential ($\Delta\psi$) or both (Hinkle and McCarty, 1978). In the acidophile, T. ferrooxidans, a natural pH gradient exists over the cell membrane with the cytoplasm maintained at about neutral (pH 5.0-6.0) and the environment, acidic (pH 1.5-3.0). Thus, a pmf is substantially contributed by a naturally existing ΔpH term (Ingledew and Cobley, 1980). Chemiosmotic theory also depends on the concept of a cell membrane which is impermeable to the movement of protons (H^+) or hydroxyl ions (OH^-) across the membrane. Protons are selectively allowed into the cell through adenosine triphosphatase (ATPase) and, thus, ATP can be formed.

Apel et al. (1980) found that the undisturbed membrane of T. ferrooxidans is selectively permeable to H^+ ions by a magnesium-dependent ATPase. In T. ferrooxidans the transfer of protons via ATPase appears to be coupled to a transfer of electrons supplied by the oxidation of Fe^{2+}. Oxygen then serves as the final electron acceptor, water is formed and a balance of protons and electrons is maintained. Concurrently, ATP is generated via the ATPase from ADP and P_i (Ingledew et al., 1977; Apel et al., 1980).

Several recent studies support the chemiosmotic theory for ATP generation via a natural pH gradient in T. ferrooxidans. These studies include the observation that protons are required for the growth of T. ferrooxidans (Apel and Dugan, 1978) and the results of experiments using various inhibitors which damage the selective

permeability of the cytoplasmic membrane (Apel and Dugan, 1978; Apel et al., 1980).

Apel and Dugan (1978) established that T. ferrooxidans requires H^+ from its environment. Unlike many other bacteria it cannot derive H^+ directly from its substrate. The initial pH of aerobic suspensions of iron-grown T. ferrooxidans was shown to increase with the addition of substrate ($FeSO_4$). This was apparently due to uptake of H^+ by the cells. The initial rise in pH was later followed in time by a net decrease in pH in the suspended media. This net acid production results from the abiotic hydrolysis of Fe^{3+} (Apel and Dugan, 1978):

$$Fe^{3+} + 3H_2O \longrightarrow Fe(OH)_3 + 3H^+$$

The uptake of H^+ from the environment by these cells was shown to be directly related to the amount of Fe^{2+} oxidized.

When solutions of dinitrophenol (DNP) or oxalacetate (which affect the permeability of the cytoplasmic membrane) were added to whole-cell suspensions of iron-grown T. ferrooxidans in the absence of $FeSO_4$ some H^+ uptake by the cells was demonstrated. Controls in which no inhibitor was added displayed no H^+ uptake by cells. This indicates that H^+ uptake in cells in the presence of $FeSO_4$ is related to the oxidation of iron and that H^+ is selectively taken up into the cells (Apel and Dugan, 1978).

Further studies have been conducted by Apel et al. (1980) using respiratory uncouplers and inhibitors on vesicles prepared from iron-grown T. ferrooxidans cultures. The vesicles were loaded with

ADP and P_i and were exposed to proton gradients comparable to the natural environment (internal pH 6-8; external pH 2-4). ATP was produced by the vesicles under these conditions. The addition of respiratory uncouplers nigericin, dicyclohexylcarbodiimide (DCCD), and pentachlorophenol all decreased the synthesis of ATP. DCCD is a specific inhibitor of the F_0 component of ATPase and allows unselective uptake of H^+ causing a destruction of the pH gradient. Pentachlorophenol is also known to adversely affect the H^+ impermeable nature of the cytoplasmic membrane resulting in destruction of the pH gradient. Nigericin is an ionophore which mediates the exchange of K^+ for H^+ leading again to a dissipation of the pH gradient. The inhibition of ATP production by these respiratory uncouplers is consistent with the theory that ATP is generated by selective uptake of H^+ via ATPase utilizing a pmf supplied by the natural pH gradient.

The addition of another ionophore, valinomycin, resulted in a net increase in ATP synthesis. Valinomycin acts by mediating an outward flux of H^+ from vesicles resulting in a decrease in the internal positive potential. The addition of this ionophore apparently led to an enhancement of H^+ uptake by the vesicles which would explain the increased ATP synthesis which was observed. The studies performed by Apel and Dugan (1978) and Apel et al. (1980) support the theory that ATP formation in acidophilic iron-grown T. ferrooxidans is a product of a pH dependent pmf.

Electrons, required by the cell to maintain a charge balance, are supplied by reduced iron and are transferred through an electron transport chain. The purification and function of electron transport carriers in T. ferrooxidans have been the subject of many papers (Din and Suzuki, 1967; Cox and Boxer, 1978; Ingledew and Cobley, 1980; Sugio et al., 1981a; Sugio et al., 1981b; Sugio et al., 1981c).

The respiratory chain must actually be considered to contain two functional directions (Ingledew and Cobley, 1980). The first includes the electron carriers which catalyze the "downhill" flow of reducing components and which function to transfer electrons from iron to molecular oxygen. The second includes the electron carriers which, at the expense of energy, catalyze "uphill" electron transport for the regeneration of reduced pyridine nucleotide ($NADH_2$). $NADH_2$ is necessary for CO_2 fixation and other biosynthetic functions (Ingledew and Cobley, 1980). The components of the "uphill" electron transport system will be included in the discussion of regeneration of reducing power in T. ferrooxidans.

The "downhill" respiratory chain links the half reactions which oxidize Fe^{2+} to Fe^{3+} and reduce molecular oxygen to water. Respiratory chain components of T. ferrooxidans have been recently resolved by potentiometric and kinetic experiments and have been described by Ingledew and Cobley (1980). These authors found two cytochromes of the *a* type, multiple cytochromes of the *c* type and two cytochromes of the *b* type. Cytochrome *b* has not been found by

other investigators using different strains of T. ferrooxidans. However, Ingledew and Cobley (1980) found this cytochrome in much lower concentrations than those of the *a* and *c* types and suggest that cytochrome *b* may not be involved with the transfer of electrons from Fe^{2+} to O_2 but rather, function in the "uphill" transfer of electrons to reduce oxidized nicotinamide adenine dinucleotide (NAD^+). Ingledew (1982) found that the concentrations of cytochromes a_1 and *c* are unusually high in T. ferrooxidans as compared to other bacteria. The author suggests that high concentrations of these electron transport chain components are necessary to catalyze the very high oxidase rates which have been observed in these bacteria.

Other components have been isolated which may be involved in the transfer of electrons from Fe^{2+} to O_2. Rusticyanin, a blue copper-containing protein first purified by Cox and Boxer (1978), has been shown to be directly reducible by Fe^{2+} with a 1:1 stoichiometry to the copper component (Cox and Boxer, 1978; Ingledew and Cobley, 1980). It has been suggested in both of these papers that rusticyanin may be the initial acceptor of electrons from Fe^{2+} during respiratory oxidation. Rusticyanin appears to be located in the periplasmic space (Ingledew and Cobley, 1980). These authors theorize that electrons may be transferred from rusticyanin in the periplasmic space to cytochrome *c*. Cytochrome *c* then shuttles between the periplasmic space and the cell membrane where it transfers electrons to cytochrome *a* which is located on the inner

surface of the membrane (Ehrlich, 1981). Cytochrome a then acts as a terminal oxidase and water is formed from transferred protons, electrons and molecular oxygen.

Sugio et al. (1981a; 1981b) have isolated two factors which appear to affect the iron-oxidizing and the cytochrome-oxidizing activities of T. ferrooxidans. The first, a copper-containing protein, which appears to stimulate both activities is located on the plasma membrane and has been called the "stimulating factor". This "stimulating factor" appears to be a protein distinct from rusticyanin. The second, cleverly called the "inhibiting factor", inhibited both activities and is probably located in the cytosol. The authors suggest a regulatory role for these two factors in iron oxidation. However, the mechanism by which regulation might occur has not been thoroughly examined.

The present model which has been presented for the generation of ATP by T. ferrooxidans is still not completely understood. However, it does suggest a manner by which these organisms can utilize a low pH environment for the generation of energy and explains the obligately acidophilic nature of these organisms. The theory has been supported by the use of traditional respiratory uncouplers both with whole cells and in prepared vesicles.

Production of Reduced Pyridine Nucleotide: The regeneration of reducing power in the form of $NADH_2$ is also dependent on the oxidation of Fe^{2+}. Electrons are transferred from Fe^{2+} via cytochrome c to NAD^+ (Aleem and Lees, 1963; Ehrlich, 1981).

Energy in the form of ATP is required to move electrons in an "uphill" direction. The cytochromes involved in the transfer of reduced substrate (i.e. Fe^{2+}) to NAD^{+} have not been as well documented as those involved in the transfer of electrons in the Fe^{2+}: O_2 couple. The respiratory chain components involved in "uphill" transfer are present in relatively lower concentrations. Ingledew and Cobley (1980) suggest that this may be attributed to the fact that only one electron appears to move in the "uphill" direction for every 20 going "downhill". Cytrochromes *c*, c_1, *b* and a flavin may be involved in the reverse electron transport system (Ingledew and Cobley, 1980; Ehrlich, 1981).

Summary: ATP generation in *T. ferrooxidans* appears to be the result of selective uptake of protons from the environment via ATPase. Energy for ATP formation is provided by a proton motive force derived primarily from a natural ΔpH gradient. This explains the obligate acidophilic nature of these organisms and the requirement of environmental protons for growth. Electrons used to balance electron-proton levels are supplied by the oxidation of inorganic cations such as Fe^{2+} and are transferred "downhill" by a series of respiratory chain components to the final electron acceptor, molecular oxygen. Rusticyanin appears to be the unique component in the electron carrier chain and functions to transfer electrons from Fe^{2+} to cytochrome *c*.

The fixation of carbon dioxide for cellular carbon requires large amounts of both energy and reduced pyridine nucleotide.

$NADH_2$ is supplied for CO_2 fixation and other synthetic processes by reverse electron transport. Electrons for the reduction of NAD^+ are provided by further oxidation of substrate and transfer "uphill" by respiratory chain components. These electron carriers are found in much smaller concentrations than those responsible for transfer "downhill". Transfer "uphill" requires energy in the form of ATP which is furnished by a pmf linked to ATPase. Thus, a mechanism is presented by which T. ferrooxidans can generate all the critical components for growth.

Apel et al. (1980) also suggest that T. ferrooxidans may, if appropriate enzymes are present, be able to utilize other oxidizable inorganic substrates to supply electrons for the system. Several authors have shown that various strains of T. ferrooxidans are capable of oxidizing other ions. These include cuprous copper (Nielsen and Beck, 1972), elemental selenium (Torma and Habashi, 1972) and uranous sulfate (DiSpirito and Tuovinen, 1981; DiSpirito and Tuovinen, 1982a; DiSpirito and Tuovinen, 1982b). These oxidations appear to proceed without the involvement of ferrous or ferric salts (Ehrlich, 1978; DiSpirito and Tuovinen, 1981). It is possible that procaryotes, such as T. ferrooxidans, may utilize many more inorganic compounds as energy sources than have been presently recognized.

Taxonomy

T. ferrooxidans was first described in 1951 by Temple and Colmer. Shortly thereafter two more iron-oxidizing bacteria were described, Ferrobacillus ferrooxidans and F. sulfooxidans (Tuovinen and Kelly, 1972; Silver, 1978). Although these three names still exist in the literature, nutritional and taxonomic studies suggested that all three types are basically the same organism and the accepted taxonomic name for these organisms became T. ferrooxidans (Tuovinen and Kelly, 1972).

Even more recently, it has been demonstrated that variations of taxonomic importance may occur among different isolates of the iron-oxidizing bacteria. Variations have been found in DNA base composition studies (Guay et al., 1976; Martin et al., 1981), physiological studies (Silver, 1978) and morphological studies (DiSpirito et al., 1982).

Guay et al. (1976) found anomalies with respect to DNA base composition of T. ferrooxidans when it was grown on different substrates. The % guanine/cytosine (% G/C) on three different substrates ranged from 54.4 to 60.1 % G/C. The authors could not fully explain the observed differences in values. However, they felt that analytical error was not responsible since three different methods which were in close agreement were used to determine the % G/C values for each sample.

Martin et al. (1981) looked at plasmid patterns of 15 different strains of iron-oxidizing T. ferrooxidans. In some strains, no plasmids were detected while other strains contained multiple plasmids of varying molecular weight and composition. When the growth substrate was changed no differences were observed in the characteristic plasmid patterns of each strain.

Physiological studies also indicate that there are differences in the organisms classified as T. ferrooxidans. Silver and Torma (1974) found that iron oxidizing bacteria which were adapted to and grown on different substrates demonstrated both quantitative and qualitative differences in their abilities to oxidize metal sulfides. In addition, differences were observed in the efficiency of carbon dioxide assimilation.

Morphological differences in T. ferrooxidans were first reported by Silverman and Rogoff (1961). They observed variations in both size and shape in cultures of T. ferrooxidans. These differences appeared to be in response to vigorous aeration by shaking. More recently, DiSpirito et al. (1982) reported differences in flagella and pili from various strains of T. ferrooxidans. The dimensions, fine structure and orientation were different from strain to strain and both polar and peritrichous flagella were observed. In addition, no flagella were detected in several of the strains examined.

The results of these types of studies support the recently proposed hypothesis (Silver, 1978) that iron-oxidizing bacteria may

not belong to one distinct species. Rather, they may comprise a metabolically similar but taxonomically distinct group of bacteria.

Summary

Since the original isolation of the iron-oxidizing thiobacilli, it has become increasingly apparent that they catalyze many biogeochemical conversions of both economic and ecological interest. Despite this fact, there are many discrepancies in the literature with respect to the growth kinetics, physiology and even morphology of these organisms. With the depletion of high-grade ores, methods of bioleaching for the recovery of valuable metals are becoming increasingly more important. It seems probable that at some time in the future genetic manipulation may be used to produce highly efficient leaching microorganisms. To be able to accomplish these goals, a more accurate and complete understanding of the growth kinetics and physiology of these organisms is essential.

METHODS AND MATERIALS

Materials

Microorganisms: Two Thiobacillus ferrooxidans isolates were used: American Type Culture Collection (ATCC) strain number 19859 and isolate AK1. AK1 was isolated from Eva Creek which is located near Fairbanks, Alaska. Eva Creek is heavily impacted by placer gold mining and is known to contain large amounts of dissolved arsenic (Brown et al., 1982). Both organisms were transferred biweekly into fresh ferrous-iron culture media and were stored at room temperature.

Growth Media: The growth medium used for both batch and continuous cultures was modified from Tuovinen and Kelly (1974) and contained: $MgSO_4 \cdot 7H_2O$ (0.4 $g \cdot l^{-1}$), $(NH_4)_2SO_4$ (0.4 $g \cdot l^{-1}$), KH_2PO_4 (0.1 $g \cdot l^{-1}$), and 10N H_2SO_4 to bring the medium to pH 1.8-2.0. Reduced iron as a sole energy source was supplied as $FeSO_4 \cdot 7H_2O$ and concentrations varied in different experiments from 5 to 30 $g \cdot l^{-1}$ for batch cultures and from 2.5 to 6.5 $g \cdot l^{-1}$ in continuous cultures. To eliminate iron-precipitate formation, the components of the medium were autoclaved in two separate solutions and were aseptically mixed when the solutions cooled to room temperature. Solution A contained the $FeSO_4$ and approximately 1-2 ml 10N H_2SO_4 per liter of final medium volume. Solution B contained the remaining salts diluted in water. The water used throughout these experiments was ASTM (American Society for Testing Standards) type I quality. When necessary, as for large

carboys of continuous culture media, sterile water was added to bring the medium to correct volume. The pH of the final medium was measured, and was adjusted to proper pH with 10N H_2SO_4 when required.

In some continuous culture experiments, 200 $mg \cdot l^{-1}$ reduced arsenic (supplied as $NaAsO_2$) was additionally incorporated into the culture media. The arsenite was dissolved in pH 2 water and was then filter-sterilized aseptically into the prepared medium.

Methods

Batch Culture: For the batch culture experiments, logarithmically growing cells were inoculated into 500 ml Erlenmeyer flasks containing iron media. The flasks were placed on a rotary shaker and were maintained at 22-23°C for the duration of each experiment. Microbiological and chemical parameters were measured periodically by aseptically removing small aliquots (generally less than 2 ml, but about 7 ml were required when dissolved organic carbon was also measured) from each flask. Triplicate flasks were used for each iron concentration so that a mean and standard error could be calculated for the measured quantities at each sampling time.

Continuous Culture: After much trial and error, a two-phase continuous culture apparatus was designed which provided the appropriate conditions for optimum iron-oxidation by the T.

ferrooxidans isolates used and which alleviated the problems of iron precipitation and bacterial growth on glassware walls reported by other investigators (D.P. Kelly, personnal communication; MacDonald and Clark, 1970). A schematic drawing of this two-phase apparatus is shown in Figure 2.

Culture medium stored in a 20 liter glass carboy was pumped by a peristaltic pump (Technicon models Varioperpex and Multiperpex, and Gilson Minipuls 2) directly into a specially designed reactor vessel. The reactor was adapted from a one liter boiling flask to which an effluent tube, air sparging tube and sampling port were added. The effluent port was placed so that the liquid volume of the flasks was maintained at 500-600 ml. The exact liquid volume (V) was measured for each flask. Compressed air to supply oxygen and carbon dioxide was added via the air port at a rate of 500-600 $ml \cdot min^{-1}$. The sampling port was sealed with a silicone stopper through which a small piece of glass tubing was inserted. A small valve connected the glass tubing to a sterile syringe. Sterile samples could be rapidly and efficiently removed by use of this sampling device. The contents of the reactor flask were continually agitated by a magnetic stirring device. The flasks were supported slighty above the stirrer to avoid any temperature impact to the culture vessel from the heat generated by the stirrer's motor. Sterile cotton plugs in the lid of the feed carboy and in the top of the reactor vessel allowed maintenance of ambient pressure throughout the system. Glass tubing and a minimal amount of silicon

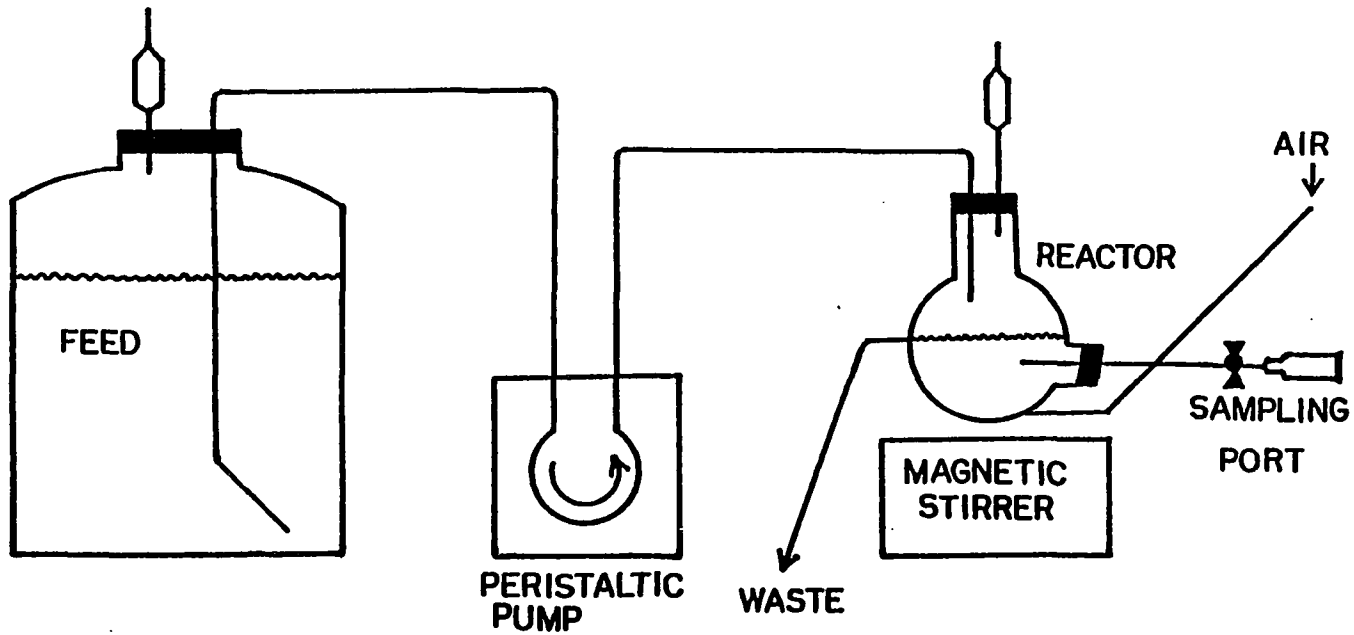

Figure 2. Two-phase continuous culture system used for cultivation of T. ferrooxidans.

tubing were used to connect the feed carboy to the reactor vessel. Surgical tubing was used in earlier systems but was eliminated after it was discovered that acid media leached substances from the tubing that inhibit bacterial growth. These inhibitors were then pumped into the reactor vessel with fresh media. The entire reactor vessel components (including all tubing and the sampling port) were autoclaved as a unit to assure sterility and were then connected aseptically to the sterile feed.

The continuous cultures were maintained in an environmentally controlled room at 22-23°C. At the onset of an experiment, 5-10 ml of logarithmically growing cells were added to 200-300 ml of fresh culture medium. The peristaltic pump was then started, the vessel filled, and continuous cultivation was begun. Generally, a slow flow was used for several days until the organisms were observed to be growing, at which time the pump was adjusted to the desired flow rate. When medium from the feed carboy was depleted or a different feed was required, a new carboy of medium could be rapidly and aseptically attached via a small quick connecting device in the feed line.

The flow rate (F, in $ml \cdot hr^{-1}$) delivered by the pump was determined by allowing the effluent to drain into a graduated cylinder for a specified amount of time (generally about 8 hours). The flow rate for each culture was measured several times to assure continuous and constant delivery by each pump at a given flow rate. The dilution rate (D) was calculated as the flow rate divided by the

volume of the culture vessel. This value, D, is equivalent to the specific growth rate of the organism (μ) at steady state (Stanier et al., 1976). After at least three residence times (R; where $R = D^{-1}$ or μ^{-1}, had passed at a given flow rate, a steady state was assumed, and samples of the reactor vessel contents were removed for microbiological and chemical analysis.

Enumeration: Cell numbers in laboratory cultures were determined by epifluorescent direct count microscopy by a method adapted from Hobbie et al. (1977). A measured aliquot (0.1-0.5 ml) of flask or reactor vessel contents was placed in a small test tube containing filtered water. Two to three drops of filtered 0.1% (in water) acridine orange solution were then added to each tube to stain the cells. Each tube of stained cell suspension was then filtered using a 3 ml syringe and Millipore Swinnex filter apparatus onto an irgalan black stained nucleopore filter (0.2 μm pore size, 25 mm diameter; Hobbie et al., 1977). Three 2 ml aliquots of filtered water were additionally filtered to rinse each tube and the filter apparatus. The damp nucleopore filter was then placed on a microscope slide with a drop of immersion oil and a cover slip and was held at 4°C in the dark for 30 minutes to one hour, after which 15-20 microscope fields were counted per slide using a Zeiss Standard microscope with an epifluorescence light source. Duplicate or triplicate filters were prepared and counted for each sample. From these data, a mean number of bacteria per ml was calculated $\pm$ 2 standard errors for a given time or dilution rate.

The acridine-orange direct-count method was selected since it allowed rapid and reproducible estimates of cell numbers. A more traditional microbiological approach of counting cells on solid media could not be used due to problems with making a suitable solid medium for growth of these organisms.

Ferrous Iron: Ferrous iron concentrations in solution (filtered through a 0.22 μm, 25 mm Millipore type HA filter) or total (nonfiltered) samples were determined by the ortho-phenanthroline spectrophotometric method of the American Public Health Association (1975). Samples were diluted to fit the standard curve linear range of 200-800 ppb, the reagents were added, and the absorbance was measured at 510 nm using a Bausch and Lomb Spectronic 2000 spectrophotometer. Ferrous iron-concentrations were additionally monitored in continuous culture media to assure chemical stability for the duration of an experiment. Ferrous iron was found to be stable in the acid media for at least 6 months.

Arsenic: Arsenic concentrations were designated as arsenic in solution (filtered through a 0.22 μm pore size, 25 mm diameter Millipore type HA filter) or as total arsenic (including arsenic in solution and associated with bacterial cells). Laboratory batch culture or continuous culture samples were collected and immediately frozen for later chemical analysis. Control experiments demonstrated stability of arsenic species in the acid culture media for greater than 6 months.

Total arsenic (ppb range) was analyzed by the graphite furnace atomic absorption method described by Wilson and Hawkins (1978) as modified with a nickel matrix (Ediger, 1975) or (ppm range) by the flame atomic absorption method using an Electrodeless Discharge Lamp (EDL) at 193.7 nm as described for the Perkin and Elmer model 4000 atomic absorption spectophotometer (Perkin-Elmer, 1982).

Arsenite ion was determined by treating 2.0 ml of sample with 0.2 ml of a modified Murphy and Riley (1962) molybdate reagent, containing 4 ml ammonium molybdate (15 g/500 ml water), 5 ml 10N H_2SO_4, 2 ml potassium antimony tartarate (0.34 g/250 ml water), and 0.108 g ascorbic acid. The samples were incubated for approximately 6 hours at which time the arsenate fraction was extracted with isoamyl alcohol. The aqueous (arsenite-containing) phase was analyzed as described above by atomic absorption spectrophotometry (Brown and Button, 1979). Arsenate ion concentrations were calculated as the difference between total arsenic and arsenite ion concentrations.

Carbon: Organic carbon was measured using the wet chemical method described for the Technicon Auto Analyzer II. (Technicon Industrial Systems, 1978). This method was selected for its sensitivity (0.4-20.0 mgC/l) and small sample-volume requirement (2 ml). Batch and continuous culture samples (5-10 ml) were collected, placed in small dichromate-rinsed vials and were immmediately frozen for carbon analysis. Media samples prepared in a similar manner

indicated that all media background carbon concentrations were negligible.

Kinetic Measurements: The following growth kinetic parameters were determined by growing T. ferrooxidans in batch culture: apparent maximum growth rate (μ_{max}); generation time (t_g); apparent maximum iron oxidation rate (V_{max}); and growth yields (Y_{carbon} and Y_{cell}). The apparent μ_{max} was determined using regression analysis of the function $\log_{10}$ cell numbers versus time. Apparent μ_{max} is equal to the slope of the growth curve at the exponential growth phase times the conversion factor 2.30 (Stanier et al., 1976). The time required for the population to double at exponential growth, t_g, was calculated using the relationship $t_g = 0.693(\mu_{max})^{-1}$ described by Stanier et al.,(1976).

The apparent maximum iron oxidation rate, V_{max}, was obtained using regression analysis of the function of iron in solution versus time during the exponential phase of growth. This calculation is based on the assumption that iron oxidized is equal to the initial ferrous iron concentration minus ferrous iron in solution.

Growth yields, Y_{carbon} and Y_{cell}, were calculated as ratios of measured carbon or cell numbers per iron oxidized at a given time. Variances reported for these parameters were estimated by the propagation of uncertainties method described by Skoog and West (1980).

A list of continuous culture parameters is found in Table 2. The relationships between these parameters were used to describe the growth and iron oxidation kinetics of T. ferrooxidans in continuous culture. Growth kinetics were described using a modified Monod (1942) model (Button, 1978) and by the Droop (1968) model. The modified Monod relationship can be expressed as

$$\mu = \mu_{max} (S-S_t) (K_\mu + (S-S_t))^{-1}$$

and can be linearized by relationship

$$S-S_t (\mu)^{-1} = K_\mu (\mu_{max})^{-1} + S-S_t (\mu_{max})^{-1}.$$

This linearization was selected rather than the more commonly used double reciprocal plot since it is less biased by deviations at low substrate concentration (Cornish-Bowden, 1979) and, therefore, yields a more accurate estimate of μ_{max}.

Iron oxidation rate (v) was analyzed as a function of ambient limiting nutrient concentration (S) and the specific growth rate. The relationship between v and S was linearized as described for the Monod model. Regression analysis was used to estimate best fit lines for all linear relationships. Time and dilution rate ($D = \mu$ at steady state) were assumed to be error free. Functions dependent on time or μ (at steady-state) were analyzed by simple linear regression. Linear functions where error was assumed in both axes were analyzed by Model II regression (Sokal and Rohlf, 1969; Ricker, 1973).

Measured chemical and physical parameters were calculated as the mean of replicate observations ± two standard errors of the mean.

Table 2. Continuous culture parameters.

Symbol	Parameter	Units
F	Flow Rate	$ml \cdot hr^{-1}$
V	Reactor Volume	ml
D	Dilution Rate ($F \cdot V^{-1}$)	hr^{-1}
R	Residence Time	hr^{-1}
S_R	Influent Limiting Nutrient Concentration	mM
S	Ambient Limiting Nutrient Concentration	mM
S_t	Threshold	mM
X	Cell Numbers	$cells \cdot ml^{-1}$
Y_{carbon}	Yield	$mg \cdot mM^{-1}$
Y_{cell}	Yield	$cells \cdot mM^{-1}$
μ	Specific Growth Rate	hr^{-1}
v	Iron Oxidation Rate	$mM \cdot hr^{-1}$
μ_{max}	Maximum Growth Rate	hr^{-1}
V_{max}	Maximum Iron Oxidation Rate	$mM \cdot hr^{-1}$
K_μ	Growth Half Saturation Constant	mM
K_{Fe}	Iron Oxidation Half Saturation Constant	mM
Q	Cell Quota	$mmol \cdot mg^{-1}$
μQ, v_{carbon}	Net Iron Oxidation Rate	$mmol(mg \cdot hr)^{-1}$
v_{cell}	Cellular Iron Oxidation Rate	$^1fmol(cell \cdot hr)^{-1}$
a_s	Steady State Affinity	$l(mg \cdot hr)^{-1}$

[1] f = femto = 10^{-15}

Variances, when ratios of measured parameters were calculated, were obtained from by the propagation of uncertainties method described by Skoog and West (1980), and were reported as the mean ± two standard deviations. Calculated error bars in all figures are within the area of the points unless otherwise noted.

RESULTS

Batch Culture

Figure 3 shows the results of a batch experiment using isolate AK1 where ferrous iron in solution, organic carbon and cell numbers were monitored over time. The initial ferrous iron concentration in the culture medium was 108 mM in this experiment. These data can be used to predict an apparent maximum specific growth rate (μ_{max}), maximum iron oxidation rate (V_{max}) and growth yields for this isolate.

The plot of $\log_{10}$ cell numbers as a function of time can be used to estimate the apparent μ_{max}. The apparent μ_{max} was calculated to be 0.089 hr^{-1} for AK1 in this experiment and in a duplicate experiment (results not shown). A similar batch culture experiment using the ATCC strain predicts an apparent μ_{max} of 0.132 hr^{-1} for this organism. Generation time (t_g), or the time required for the population to double during exponential growth, was found to be approximately 7.7 hr for AK1 and 5.3 hr for ATCC.

The relationship between ferrous iron in solution and time was used to estimate an apparent maximum iron oxidation rate (V_{max}). Apparent V_{max} was found to be 3.34 mmol $(l \cdot hr)^{-1}$ the AK1.

The organic carbon and cell number data were used to calculate growth yields, Y_{carbon} and Y_{cell}, for AK1. Y_{carbon} ranged from 0.12 to 0.20 mg carbon (mmol Fe^{2+} oxidized)$^{-1}$ while

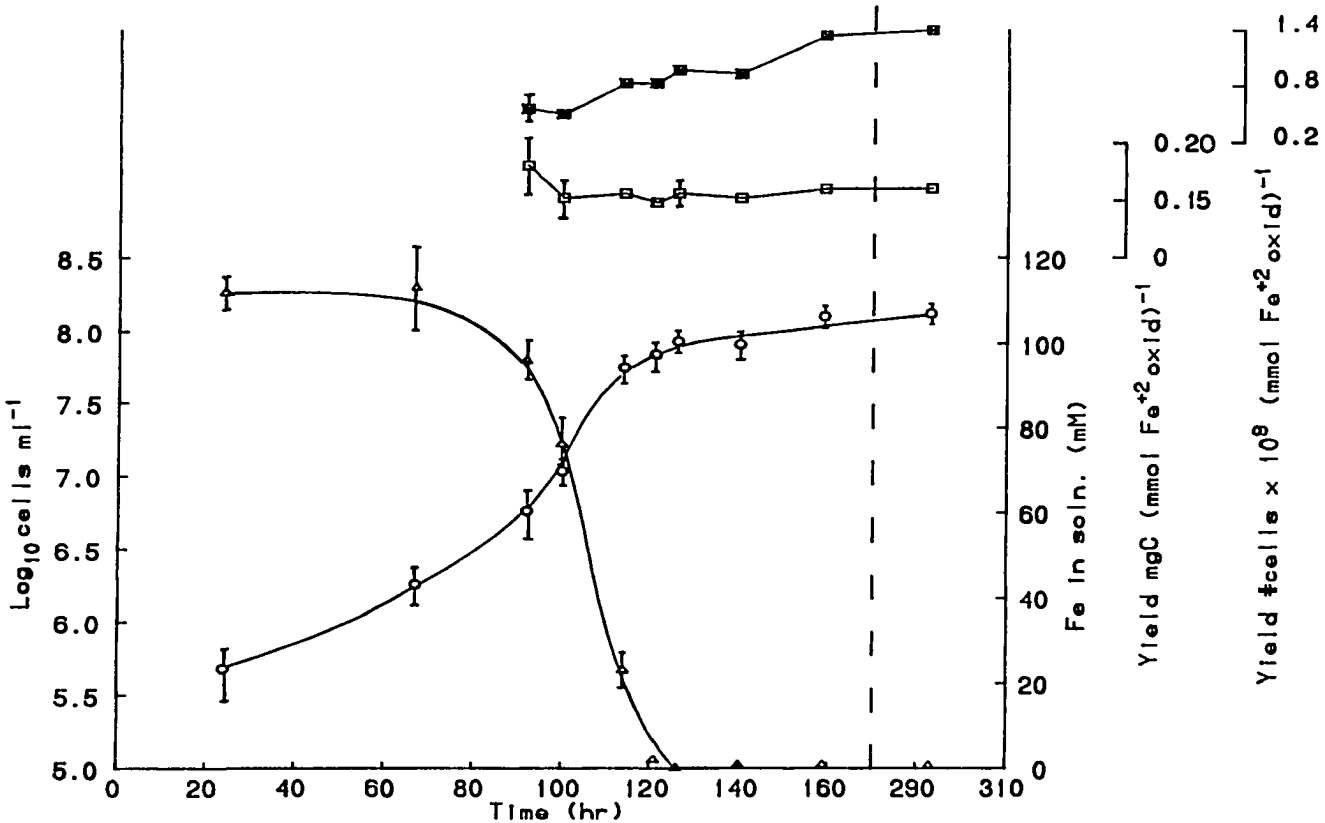

Figure 3. Ferrous iron in solution (△), $\log_{10}$ cell numbers (○), Y_{carbon} (□), and Y_{cell} (⊠) as functions of time for batch grown T. ferrooxidans, isolate AK1.

Y_{cell} demonstrated an increase over time from 0.25 to $1.20 \cdot 10^9$ cells (mmol Fe^{2+} oxidized)$^{-1}$. In a duplicate experiment using the AK1 isolate (results not shown), a similar final Y_{cell} value of $1.3 \cdot 10^9$ cells (mmol Fe^{2+} oxidized)$^{-1}$ was calculated. These Y_{carbon} and Y_{cell} values suggest that carbon per cell is decreasing over time in batch grown cells. At the stationary phase of growth (near the end of the experiment), there appears to be approximately 0.12 pico gram (pg) carbon per cell.

Continuous Culture

Physiological and Chemical Data: Physiological and chemical data for iron-limited continuous cultures of *T. ferrooxidans* AK1 and ATCC are found in Tables 3 and 4, respectively. As indicated in Table 3, some of the AK1 cultures were also supplied with 1.33 or 2.67 mM arsenite added to the feed. Arsenic speciation and quantitation indicates that the arsenite was not oxidized in the presence of isolate AK1 in these continuous cultures. From these physiological and chemical data, the iron-limited growth kinetics of isolate AK1 can be described. Some of the kinetic parameters calculated for AK1 can also be compared to those found for the less extensively studied ATCC strain.

Modified Monod Growth Kinetics: Figure 4 shows the relationship between the cellular iron oxidation rate (v_{cell}) and external ferrous iron concentration (S) for isolate AK1. The x-intercept of

Table 3. Steady-state physiological and chemical data for iron-limited continuous cultures of T. ferrooxidans, isolate AK1. Data points are listed as the mean ± two standard errors.

Fe^{2+}_{feed} (mM)	As^{3+}_{feed} (mM)	μ (hr^{-1})	Fe^{2+}_{soln} (mM)	As^{3+}_{soln} (mM)	Carbon ($mg \cdot l^{-1}$)	Cells Numbers ($cells \cdot 10^{7} \cdot ml^{-1}$)
9.0	---	0.006	0.33±0.00	---	3.4±0.49	1.02±0.11
9.0	---	0.011	0.32±0.00	---	3.2±0.15	1.36±0.14
9.0	---	0.013	0.36±0.01	---	2.6±0.29	0.76±0.12
9.0	---	0.026	0.48	---	---	---
9.0	---	0.030	1.12±0.02	---	1.9±0.31	0.58±0.04
9.0	---	0.038	0.89±0.07	---	1.8±0.15	0.87±0.09
9.5	---	0.008	0.36	---	3.7±1.40	---
9.0	1.33	0.012	0.42	---	---	---
9.0	1.33	0.034	1.00	---	1.5±0.01	---
9.0	2.67	0.007	0.30±0.11	2.64±0.10	4.4±0.53	1.10±0.12
9.0	2.67	0.014	0.45±0.00	2.64±0.52	3.3±0.38	0.97±0.12
9.0	2.67	0.030	0.76±0.09	2.79±0.32	2.0±0.04	0.96±0.10
9.0	2.67	0.034	1.20±0.04	2.73±0.25	1.9±0.15	0.70±0.10
17.9	---	0.033	1.45±0.08	---	2.9±0.11	1.23±0.16
22.4	---	0.016	0.64	---	4.9±1.00	---
22.4	---	0.027	1.05	---	5.4±4.01	---
22.4	---	0.044	1.68		3.0±0.40	---
22.4	---	0.050	2.19	---	---	---
23.3	---	0.028	1.00		4.1±0.40	---
23.3	---	0.052	1.54	---	---	---

Table 4. Steady-state physiological and chemical data for iron-limited continuous cultures of T. ferrooxidans, strain ATCC. Data points are listed as the mean ± two standard errors.

Fe^{2+}_{feed} (mM)	μ (hr^{-1})	Fe^{2+}_{soln} (mM)	Carbon ($mg \cdot l^{-1}$)	Cell Numbers ($cells \cdot 10^7 \cdot ml^{-1}$)
9.0	0.080	0.88±0.09	1.0±0.27	---
9.0	0.121	1.08±0.02	1.1±0.10	---
17.9	0.021	0.82±0.04	2.3±0.24	1.93±0.00
17.9	0.033	0.76±0.02	2.1±0.11	1.94±0.25
17.9	0.045	0.89±0.16	2.3±0.20	2.82±0.37
17.9	0.049	1.20±0.01	1.4±0.39	1.14±0.11
17.9	0.062	1.02±0.03	2.4±0.52	2.52±0.89
17.9	0.078	0.97	2.0±0.34	2.46±0.66
17.9	0.112	3.83±0.50	1.6±0.60	1.08±0.30

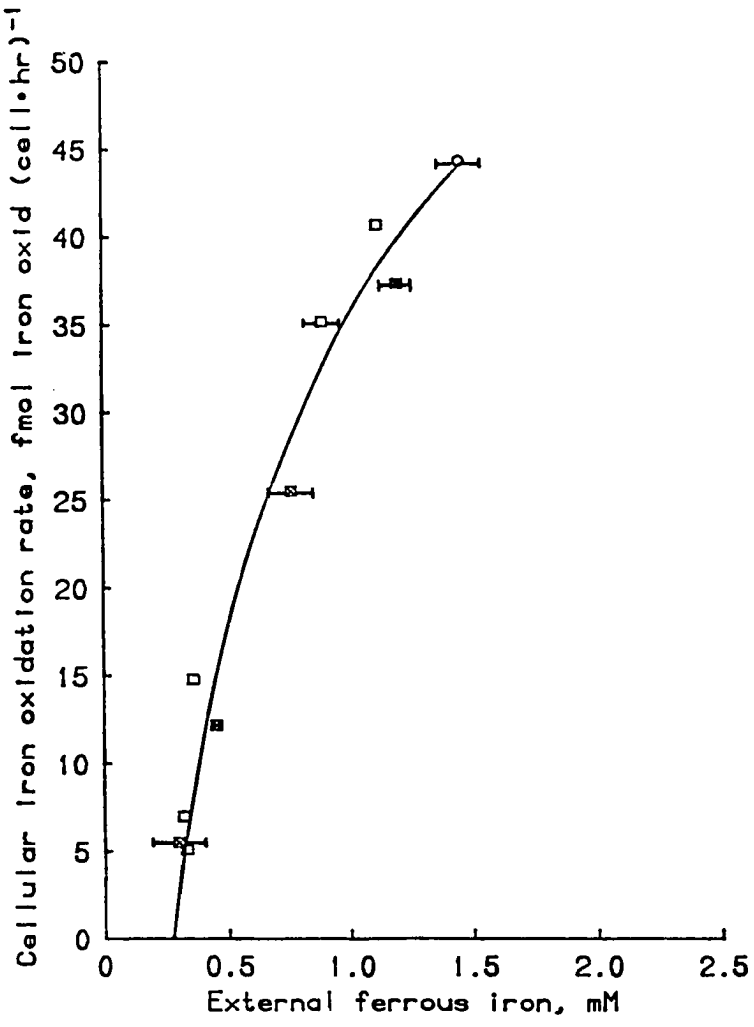

Figure 4. Steady state cellular iron oxidation rate (v_{cell}) as a function of external ferrous iron concentration (S) for T. ferrooxidans, isolate AK1. Threshold ferrous iron concentration (S_t) was estimated to be 0.25 mM for this isolate. The symbols represent different influent ferrous iron concentrations (S_R): 9.0-9.5 mM (□), 9.0 mM + 1.3-2.7 mM As^{+3} (⊠) and 17.9 mM (○). The line shown is hand drawn.

this graph was used to estimate an apparent threshold (S_t = 0.25 mM). The threshold concentration includes ferrous iron required for cell maintenance in the absence of growth and ferrous iron which might be unavailable to the organism for energy utilization (Button, 1978). Although insufficient continuous culture data are available to describe a complete kinetic profile for the ATCC strain, there appears to be a threshold ferrous iron concentration of approximately 0.6 mM for this strain. The concept of threshold can be applied to a modified Monod (1942) growth curve as shown in Figure 5. A linearized form of the Monod growth curve is found in Figure 6 and was used to estimate an apparent μ_{max} of 0.054 ± 0.020 hr^{-1} and a half saturation constant (K_μ) of 0.50 mM for strain AK1 (Cornish-Bowden, 1979). There is no apparent effect on the Monod growth kinetics of strain AK1 when initial iron concentration was varied or when arsenite was added to the ferrous iron-containing feed.

Droop Growth Kinetics: Droop (1968) growth kinetics can be used to predict an apparent μ_{max} based on internal nutrient or substrate concentrations referred to as cell quota (Q), as opposed to the Monod model in which kinetic growth parameters are calculated as a function of the external substrate concentration (S). Although ferrous iron is not transported internally by I. ferrooxidans, a parameter analogous to Q can be calculated based on ferrous iron oxidized. The parameter μQ represents the net steady state ferrous iron oxidation rate (also referred to as v_{cell}). The linearized

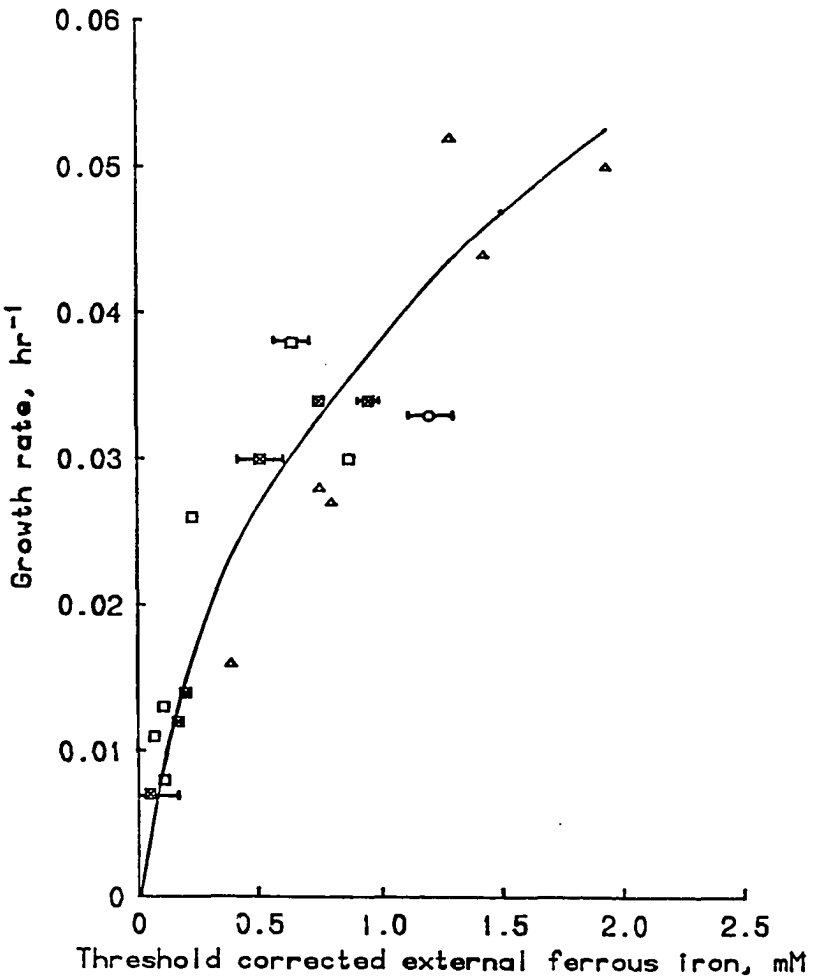

Figure 5. Steady state growth rate (μ) as a function of threshold corrected external ferrous iron concentration ($S-S_t$) for T. ferrooxidans, isolate AK1. The symbols represent different influent ferrous iron concentrations (S_R): 9.0-9.5 mM (□), 9.0 mM + 1.3-2.7 mM As^{+3} (⊠), 17.9 mM (○) and 22.4-23.3 mM (△).

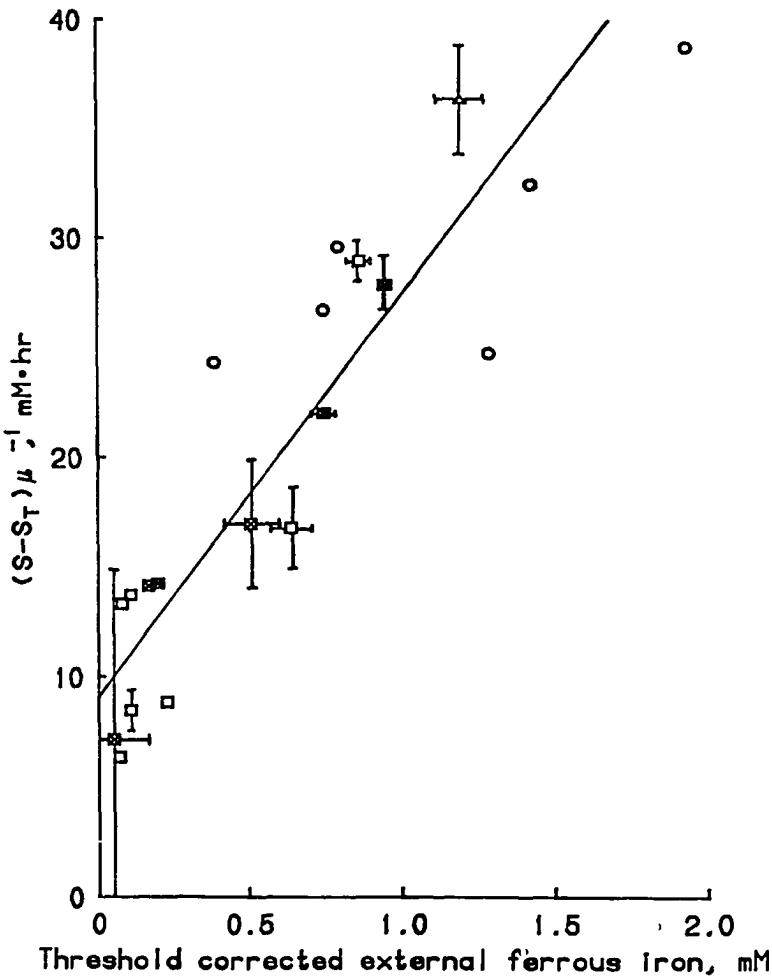

Figure 6. Linearized form of the Monod growth curve for I. ferrooxidans, isolate AK1. The equation for the line using Model II regression is y = 18.38 + 9.04. The 95% confidence limits for the slope are 14.13 to 23.78. The symbols represent different influent ferrous iron concentrations (S_R): 9.0-9.5 mM (□), 9.0 mM + 1.3-2.7 mM As^{+3} (⊠), 17.9 mM (△) and 22.4-23.3 mM (○).

form of the Droop equation is found in Figure 7 (Rhee, 1980). An apparent μ_{max} of 0.056 is estimated from the slope of the "μ-Q" versus "Q" plot (Figure 7) for strain AK1. Again, using Droop kinetics, no apparent effect was observed on kinetic growth parameters when influent ferrous iron concentration was varied or when arsenite was added to the culture media.

Growth Yields: Figure 8 shows the steady state relationship between Y_{carbon} and specific growth rate for iron-limited continuous cultures of T. ferrooxidans isolate AK1. Cell yield is defined as the unit cell mass produced per substrate consumed (Stanier et al., 1976). If cellular carbon is assumed to be approximately 50% of the dry weight of a cell (Stanier et al., 1976), then the plotted values represent approximately one half the cell yield on a dry weight basis. In contrast to the AK1 isolate, Y_{carbon} did not vary significantly with growth rate for the ATCC strain. Y_{carbon} varied randomly from 0.11 to 0.15 mg (mmol Fe^{2+} oxidized)$^{-1}$ over a steady state growth rate range of 0.021 to 0.121 hr^{-1} for ATCC.

Estimation of Carbon Per Cell: The steady state iron oxidation rates per cell (v_{cell}) and per unit carbon (μQ or v_{carbon}) as functions of specific growth rate are found in Figures 9 and 10, respectively, for isolate AK1. If the slopes of the best-fit lines for the two plots are compared, an estimate of average gram carbon per cell can be calculated. This value is approximately 0.18 pg carbon per cell for isolate AK1. This gram carbon per cell value

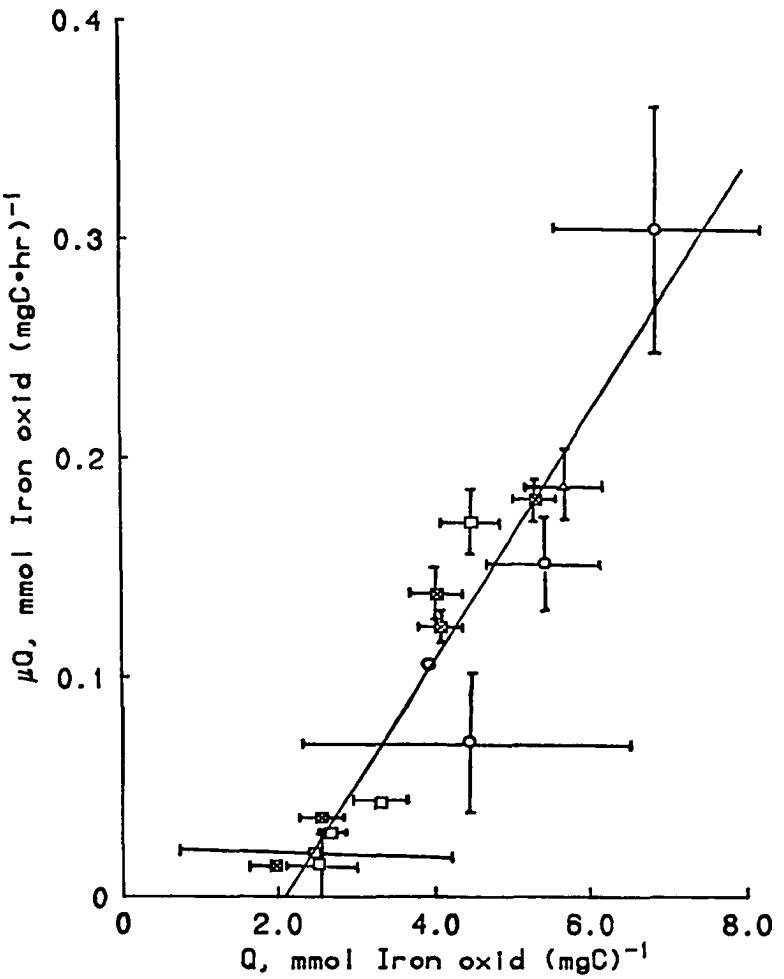

Figure 7. Net steady state ferrous iron oxidation rate (μQ or v_{carbon}) as a function of "cell quota" (Q) for T. ferrooxidans, isolate AK1. The equation for the line using Model II regression is y = 0.056x - 0.118. The 95% confidence limits for the slope are 0.046 to 0.066. The symbols are the same as in Figure 6.

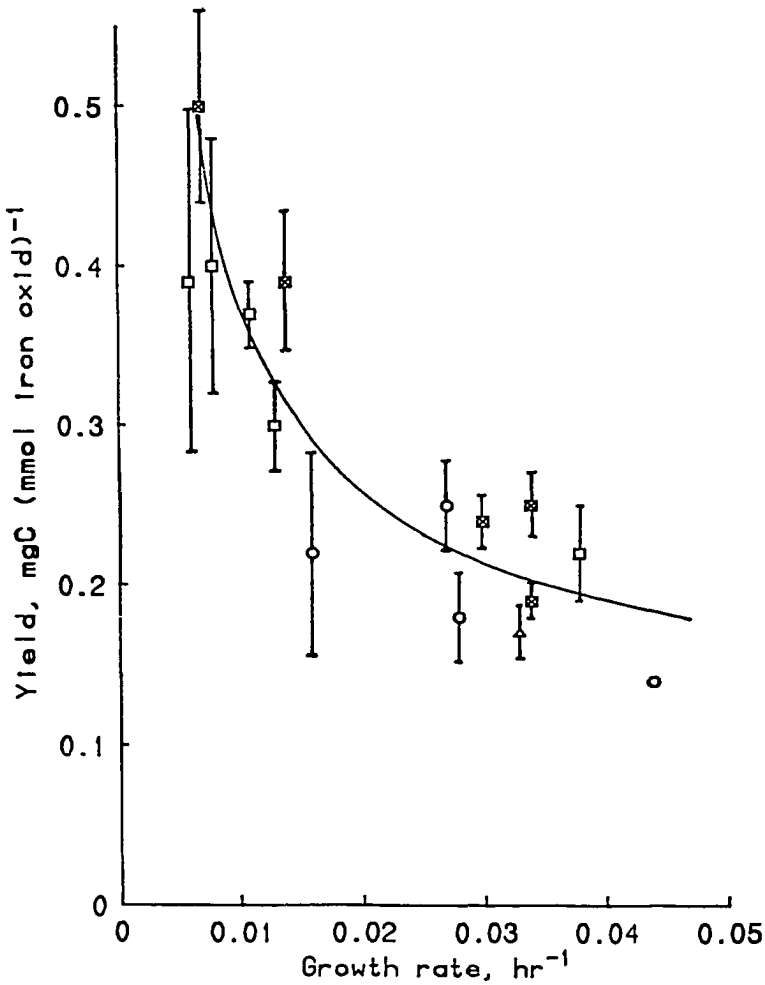

Figure 8. Steady state yield of cellular carbon (Y_{carbon}) as a function of growth rate (μ) for T. ferrooxidans, isolate AK1. The symbols are the same as in Figure 6. The line shown is hand drawn.

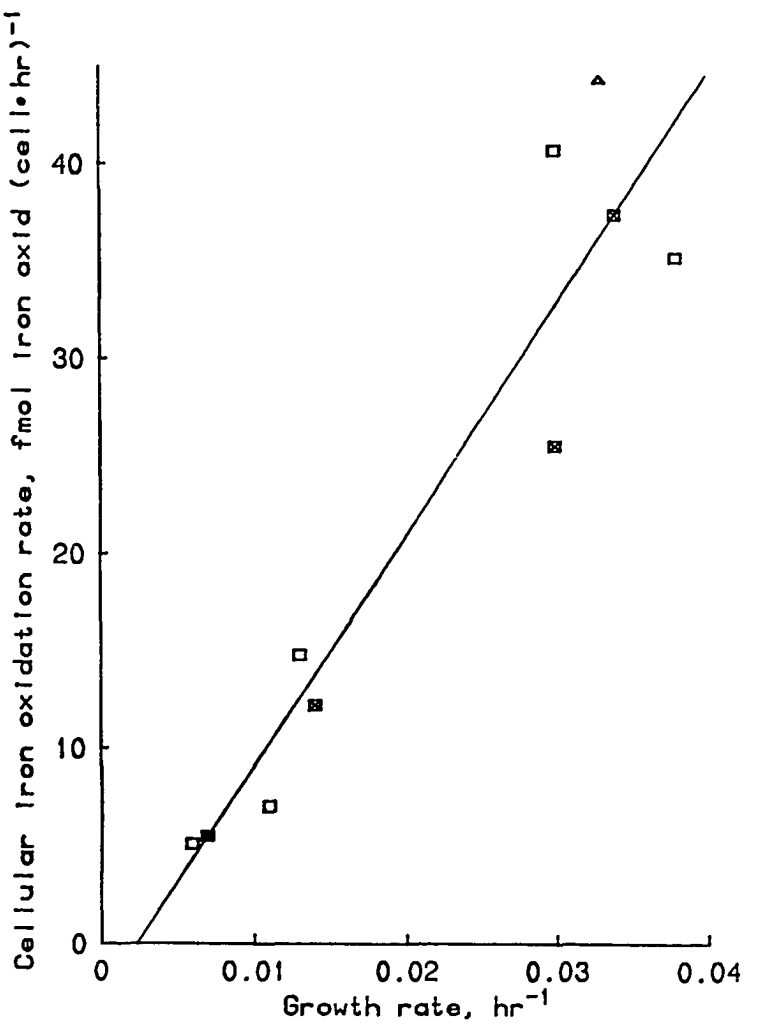

Figure 9. Steady state cellular iron oxidation rate (v_{cell}) as a function of growth rate (μ) for T. ferrooxidans, isolate AK1. The equation for the line at the 95% confidence level using simple linear regression is $y = (1.18 \pm 0.34 \cdot 10^3)x - (2.76 \pm 8.46)$ with $r = 0.94$. The symbols are the same as in Figure 6.

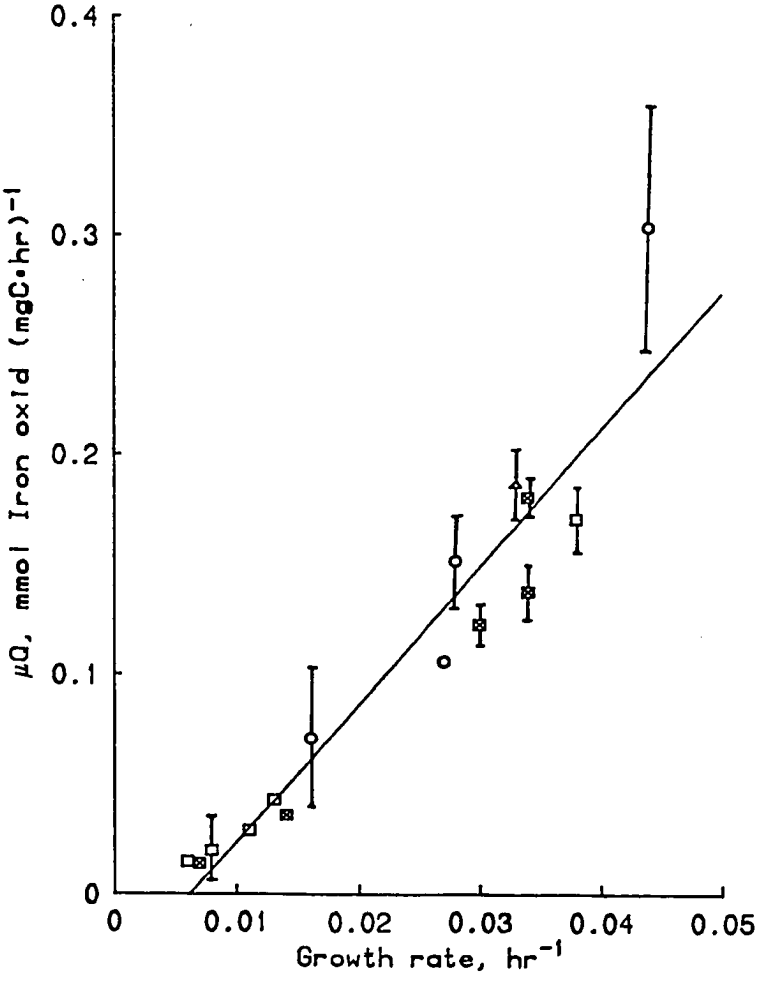

Figure 10. Steady state iron oxidation rate per organic carbon (μQ or v_{carbon}) as a function of dilution rate (μ) for I. ferrooxidans, isolate AK1. The equation for the line at the 95% confidence level using simple linear regression is y = (6.25±1.20)x - (0.04±0.04) with r = 0.95. The symbols are the same as in Figure 6.

only indicates an average value for strain AK1. The individual carbon per cell ratios as a function of specific growth rate show a trend toward decreasing values as the specific growth rate increases. These values range from 0.38 to 0.16 pg carbon per cell. The ratio of carbon per cell calculated as a function of specific growth rate for the ATCC strain ranges randomly from 0.07 to 0.14 pg per cell and appears to be independent of the specific growth rate. If carbon is assumed to be approximately 50% of the cellular dry weight, then the cell dry weights are in the range of 0.32 to 0.76 for AK1 (dry weight is a function of μ), and 0.14 to 0.23 pg dry weight per cell for ATCC (dry weight does not appear to be a function of μ).

Iron Oxidation Kinetics: Figure 11 shows the Michaelis-Menten relationship between the gross iron oxidation rate (v) and threshold corrected external ferrous iron concentration ($S-S_t$) for three different influent ferrous iron concentrations (S_R). A linearized form of this equation is found in Figure 12 (Cornish-Bowden, 1979). Figure 12 can be used to predict a maximum iron oxidation rate (V_{max}) and half saturation constant (K_{Fe}) for each value of S_R. The apparent V_{max} predicted from the reciprocal slope of the best fit lines for S_R = 9.0 mM is 0.383 mmol $(l \cdot hr)^{-1}$ and for S_R = 22.4-23.3 mM is 1.901 mmol $(l \cdot hr)^{-1}$. The half saturation constant, K_{Fe}, estimated from V_{max} times the y-intercept was found to be 0.347 and 1.543 mM for S_R = 9.0 and S_R = 22.4-23.3 mM, respectively. There were insufficient data

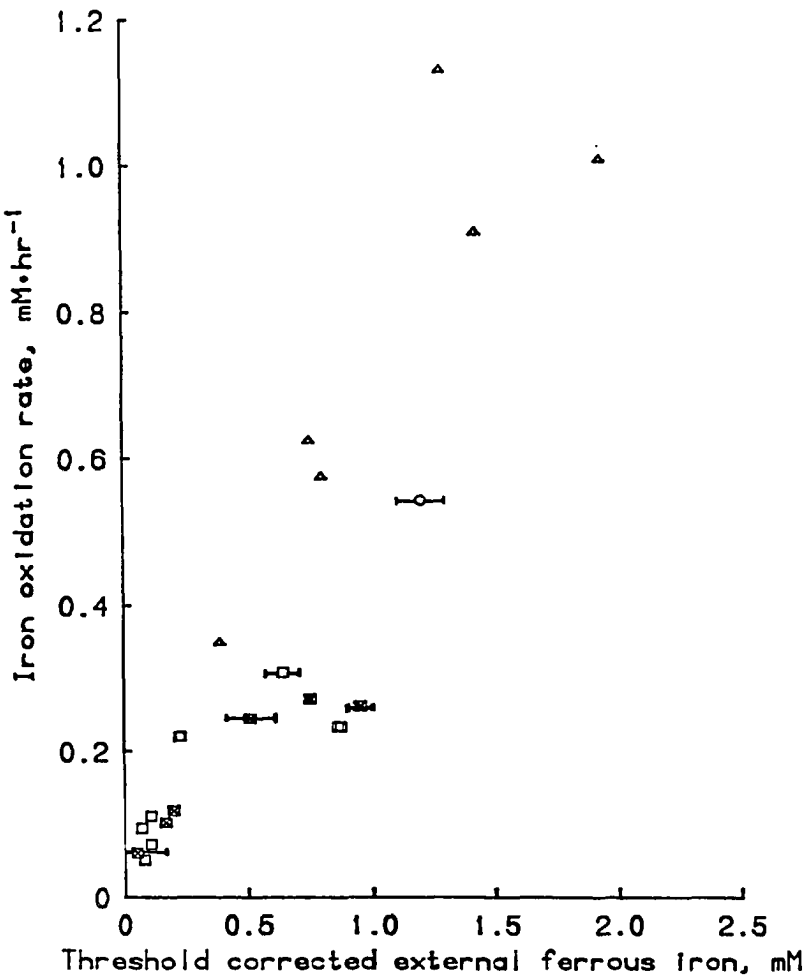

Figure 11. Steady state ferrous iron oxidation rate (v) as a function of threshold corrected external ferrous iron concentration ($S-S_t$) at different influent ferrous iron concentrations (S_R) for T. ferrooxidans, isolate AK1. The symbols are the same as in Figure 5.

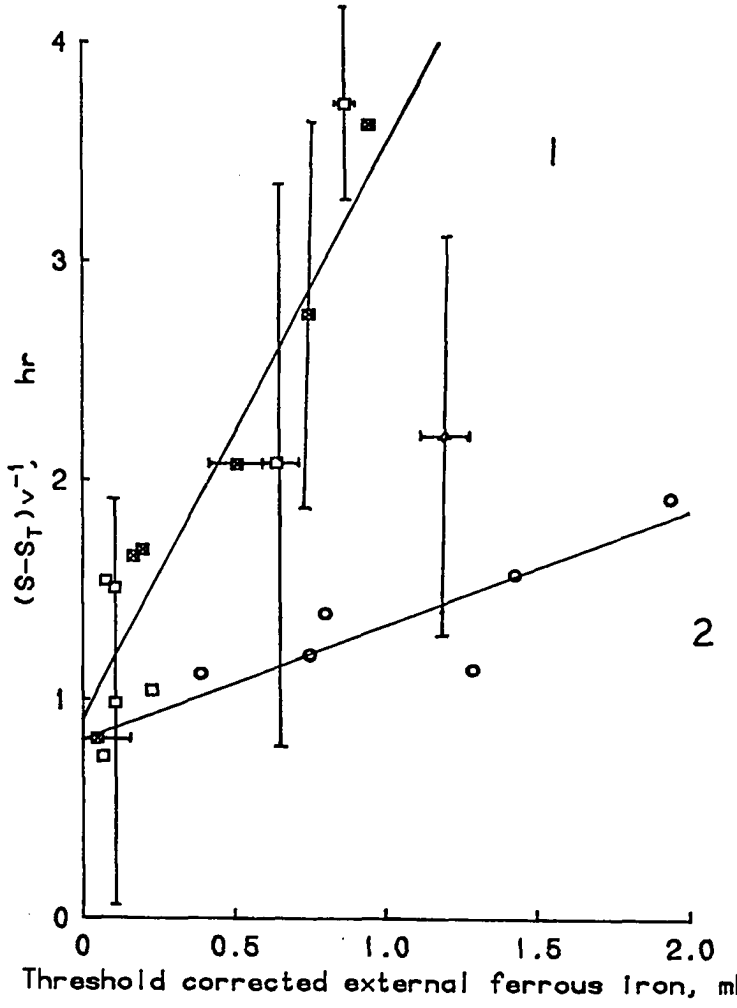

Figure 12. Linearization of the steady state relationship between ferrous iron oxidation rate (v) and threshold corrected ferrous iron concentration ($S-S_t$) at three different influent ferrous iron concentrations (S_R) for *T. ferrooxidans*, isolate AK1. The equations for the lines using Model II regression are: 1. $y = 2.614x + 0.907$ with 95% confidence limits for the slope of 1.778 to 3.297 and 2. $y = 0.526x + 0.811$ with 95% confidence limits for the slope of 0.043 to 0.886. The symbols are the same as in Figure 6.

available to predict these parameters for S_R = 17.9 mM. The gross iron oxidation rate can thus be shown to be a function of S_R.

Figure 13 shows the relationship between dissolved organic carbon and threshold corrected external ferrous iron as a function of S_R for isolate AK1. Since the calculated error associated with the x axis is negligible in comparison to the error associated with the y axis, the equations for the lines shown were predicted using simple linear regression. Total organic carbon production is shown to be dependent on influent substrate concentration and on threshold corrected external ferrous iron concentration (ambient substrate concentration). Increased organic carbon production at increasing influent substrate concentration implies greater cell biomass and, therefore, greater ferro-oxidase activity.

Figure 14 shows the Michaelis-Menten relationship between the net iron oxidation rate (v_{carbon} or μQ) and threshold corrected external ferrous iron concentration. Although a theoretical maximum net iron oxidation rate must exist, this graph does not indicate an asymptote. Therefore, the net iron oxidation rates observed for AK1 must be significantly below maximal. There is no apparent effect on the net iron oxidation rate as a function of $S-S_t$ when the initial iron concentration was varied or when arsenite was added to the feed containing ferrous iron.

Steady state affinity (a_s) for a growth rate limiting substrate can be used as an estimate of an organism's ability to compete for that substrate. The slope of the best fit line of net

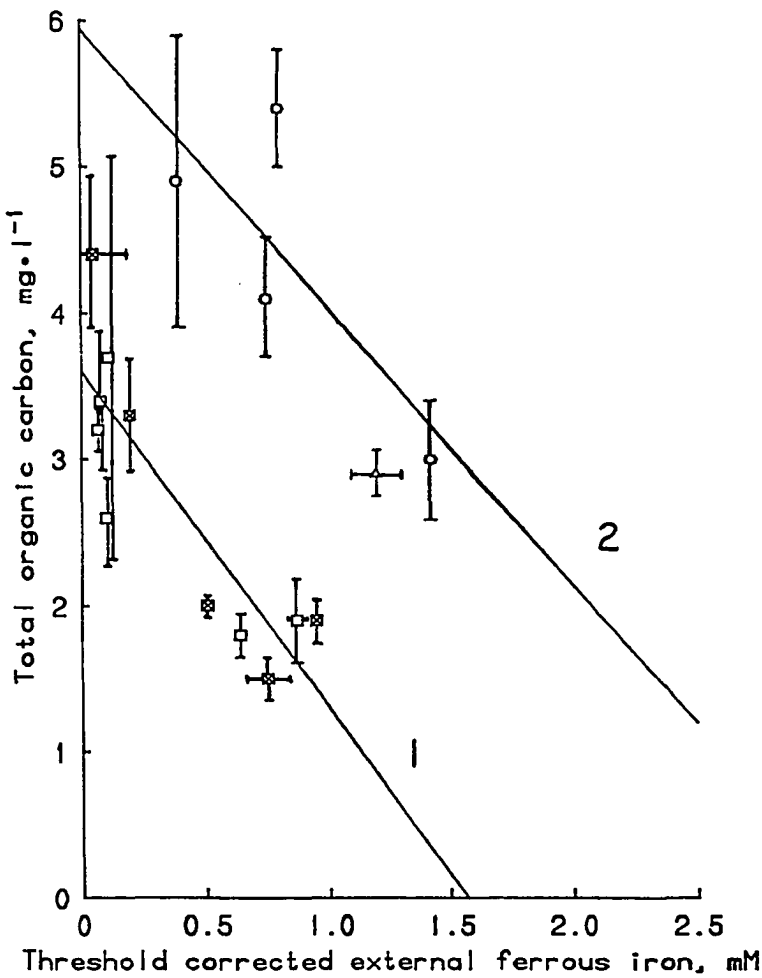

Figure 13. Steady state organic carbon as a function of threshold corrected external ferrous iron concentration ($S-S_t$) at different influent ferrous iron concentrations (S_R) for I. ferrooxidans, isolate AK1. The equations for the lines at the 95% confidence level using simple linear regression are: 1. $y = (-2.30\pm1.03)x + (3.60\pm0.53)$ with $r = -0.86$ and 2. $y = (-1.90\pm4.55)x + (5.95\pm4.19)$ with $r = -0.78$. The symbols are the same as in Figure 6.

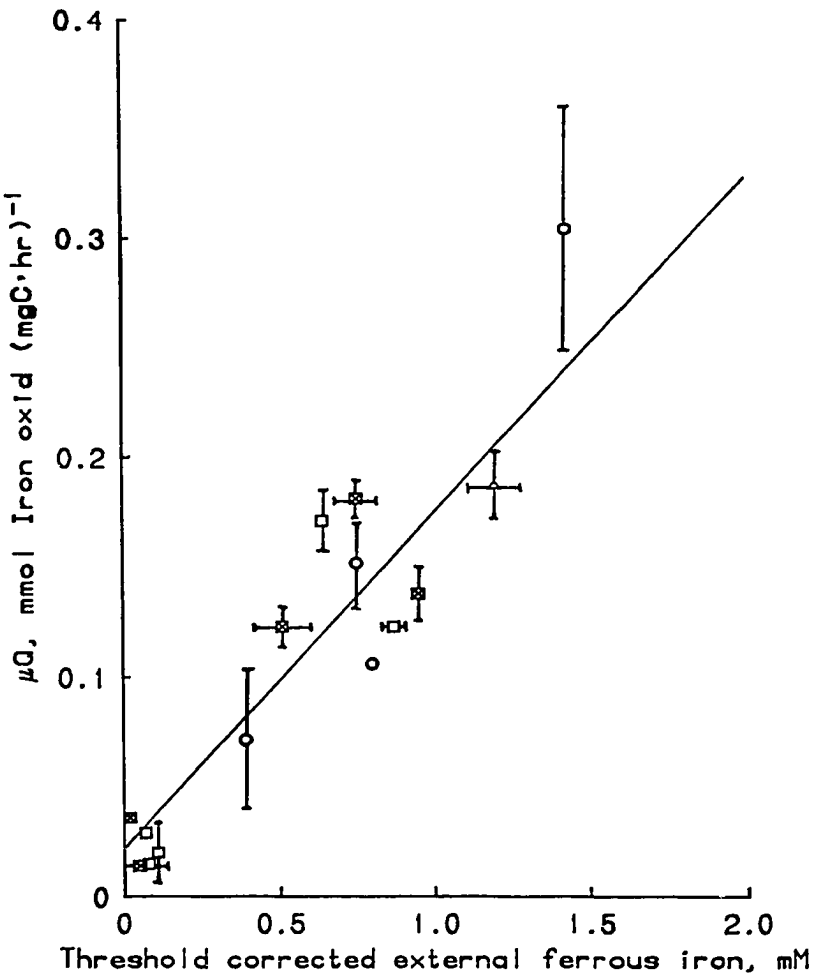

Figure 14. Steady state net iron oxidation rate (v_{carbon} or μQ) as a function of threshold corrected external ferrous iron concentration ($S-S_t$) at different influent ferrous iron concentrations (S_R) for T. ferrooxidans, isolate AK1. The equation for the line using Model II regression is $y = 0.153x + 0.022$. The 95% confidence limits for the slope are 0.114 to 0.172. The symbols are the same as in Figure 6.

iron oxidation rate versus $S-S_t$ (Figure 14) can be used as an approximation of steady state affinity (Button, 1978). From Figure 14, the a_s for strain AK1 is approximately 0.153 l $(mgC \cdot hr)^{-1}$.

The best fit line for Figure 14 would be expected to go through the origin (Button, 1978). The Model II regression method (Sokal and Rohlf, 1969) used to calculate the best fit line only predicts a confidence interval around the ratio estimator (slope). However, if the error in the x variable ($S-S_t$) is assumed to be negligible (see Figure 14), a simple linear regression can be used to predict a best fit line. This equation at the 95% confidence level is $y = (0.174 \pm 0.04)x + (0.010 \pm 0.027)$. The equation predicted using simple linear regression indicates that the true line may pass through the origin if the error in the x value can be assumed to be negligible.

DISCUSSION

Growth Kinetics

Tables 5 and 6 contain summaries of growth kinetic parameters calculated from batch and continuous culture experiments for T. ferrooxidans isolate AK1 and strain ATCC. Estimates of values such as apparent μ_{max} indicate that the growth kinetics differ in nonsteady state (batch culture) versus steady state (continuous culture) conditions. Such results reflect the transient nature of batch cultures which switch from iron non limiting to iron limiting during the growth cycle. Continuous cultures, in contrast, maintain steady state iron-limited growth.

The apparent μ_{max} predicted from batch culture data for AK1 is the highest observed for this isolate. This same phenomenon has been observed for other microorganisms. Lang (1980) suggests that different metabolic mechanisms may control cell nutrient levels and transport rates when transient rather than steady state conditions exist. Apparent maximum specific growth rates calculated for AK1 in both batch and continuous cultures were lower than those reported for other T. ferrooxidans strains (see Table 1) and lower than the value estimated for the ATCC strain in batch culture. Insufficient data were available to accurately assess μ_{max} for the ATCC strain in continuous culture. However, μ_{max} must exceed the measured specific growth rate (dilution rate) of 0.121 hr^{-1} for this strain

Table 5. Summary of growth kinetic parameters calculated for *T. ferrooxidans* isolate AK1 and strain ATCC grown in batch cultures.

Isolate	Parameter	Value
AK1	μ_{max}	0.089 hr^{-1}
AK1	t_g	7.7 hr
AK1	Y_{carbon}	0.12-0.20 mgC $(mmol\ Fe)^{-1}$
AK1	Y_{cell}	$1.20\text{-}1.30 \cdot 10^9$ cells $(mmol\ Fe)^{-1}$
AK1	[1]Carbon Per Cell	0.12 $pg \cdot cell^{-1}$
ATCC	μ_{max}	0.132 hr^{-1}
ATCC	t_g	5.3 hr

[1] Final value calculated at the end of an experiment (stationary phase of growth).

Table 6. Summary of growth kinetic parameters calculated for T. ferrooxidans isolate AK1 and strain ATCC grown in continuous cultures.

Isolate	Parameter	Value
AK1	$^{1}\mu_{max}$	0.054 hr^{-1}
AK1	$^{2}\mu_{max}$	0.056 hr^{-1}
AK1	$^{1}K_{\mu}$	0.50 mM
AK1	S_t	0.25 mM
AK1	Y_{carbon}	Function of μ (Figure 8)
AK1	^{3}Carbon Per Cell	0.16-0.38 $pg \cdot cell^{-1}$
ATCC	S_t	0.6 mM
ATCC	Y_{carbon}	0.11-0.15 mgC $(mmol\ Fe)^{-1}$
ATCC	^{3}Carbon Per Cell	0.07-0.14 $pg \cdot cell^{-1}$

[1] Calculated using the modified Monod model.
[2] Calculated using the Droop model.
[3] Range of values calculated for all measured growth rates.

(Table 4). It is clear from published values (Table 1) and from these studies that the growth kinetics of microorganisms classified as T. ferrooxidans are variable from strain to strain. These apparent strain differences complicate comparisons of published kinetic data.

The steady state growth kinetics of isolate AK1 can be described by either the modified Monod (external) model or by the Droop (internal) model. Both models predict a similar μ_{max} for AK1 under steady state conditions. The modified Monod (Button, 1978) model indicates a threshold external ferrous iron concentration of 0.25 mM and approximately 0.6 mM for AK1 and ATCC, respectively. Threshold values have not been described for other strains of T. ferrooxidans grown in continuous culture (MacDonald and Clark, 1970; Tomizuka et al., 1976; Guay et al., 1977; Jones and Kelly, 1977; Kelly et al., 1977; Kelly and Jones, 1978; Hoffman et al., 1981).

The value of the half saturation constant, K_{μ}, (estimated from a linearization of the Monod growth curve) is approximately 0.5 mM for AK1. This value was corrected for a threshold ferrous iron concentration of 0.25 mM. Published half saturation constants vary widely from 0.7- 37 mM (Table 1). This variability is probably the result of differences in strains, physical culturing conditions (such as temperature and pH), and difficulties reported in growing T. ferrooxidans in continuous culture (MacDonald and Clark, 1970; Kelly et al.,1977).

Kelly et al. (1977) used continuous cultures to study the iron-limited growth kinetics of one strain of T. ferrooxidans. These authors concluded that the growth of this strain was subject to a complex non competitive or competitive inhibition of ferrous iron oxidation. This inhibition appeared to depend on the ferrous iron concentration in the medium feed. Product inhibition in this strain resulted in "premature washout" at apparent critical dilution rates. The organism was also able to maintain a steady state dilution rate of up to 1.33 hr^{-1} at influent ferrous iron concentrations of 9-25 mM. This dilution rate ($D = \mu$ at steady state) is much higher than that observed for other strains in other continuous culture studies (see Table 1) and is at least one order of magnitude greater than that observed for AK1 or ATCC. Additionally, my data show no evidence of product inhibiton on the growth kinetics of AK1 or ATCC when the influent ferrous iron concentration was varied from 9.0-23.3 mM.

Yield (Y_{carbon}) for AK1 was relatively constant in batch grown cultures ranging from 0.12 to 0.20 mg carbon (mmol iron oxidized)$^{-1}$. However, steady state (continuous culture) data indicate a decreasing curvilinear dependence of Y_{carbon} on the specific growth rate (Figure 8). The steady state carbon per cell ratio also appears to decrease with increasing dilution rate for isolate AK1. This indicates that the observed decrease in Y_{carbon} as dilution rate increases is a result of the decline in cell carbon rather than a decline in cell numbers. The highest observed

Y_{carbon} for AK1 is 0.5 mg $(mmol)^{-1}$ at a specific growth rate of 0.007 hr^{-1}. In contrast, Y_{carbon} varies randomly in the ATCC strain from 0.08 to 0.15 mg $(mmol)^{-1}$ over a steady state growth rate range of 0.021-0.121 hr^{-1}. The carbon per cell ratio does not appear to be a function of dilution rate for strain ATCC. Since Y_{carbon} and carbon per cell were not measured at very low growth rates for ATCC, the significance of this apparent difference cannot be fully evaluated. However, at equivalent dilution rates the carbon per cell ratio is always larger for AK1 than for ATCC. If carbon is assumed to be approximately 50% of the cell dry weight, then the observed carbon per cell difference for the two isolates indicates that the average cell size (biomass) is greater for AK1 than for ATCC.

Iron Oxidation Kinetics

The gross ferrous-iron oxidation rate (v) at steady state is dependent upon the influent ferrous iron concentration as shown in Figures 11 and 12 for AK1. The maximum iron oxidation rate (v_{max}) and the half saturation constant (K_{Fe}) both appear to increase with increasing influent ferrous iron concentration (S_R). The half saturation constant (K_{Fe}) is 0.347 mM at S_R = 9.0-9.5 mM and is 1.543 mM at S_R = 22.4-23.3 mM. The apparent V_{max} is 0.383 and 1.901 mM hr^{-1} for S_R = 9.0-9.5 mM and 22.4-23.3 mM,

respectively. This phenomenon was also observed by Guay et al. (1977).

Guay et al. (1977) examined the steady state oxidation kinetics of a T. ferrooxidans strain at influent ferrous iron concentrations of 89-161 mM. The results of their study indicate that even at an S_R of 161 mM the maximum iron oxidation capacity of the strain investigated was not obtained.

The observed dependence of V_{max} on influent substrate concentration is predicted from Michaelis-Menten kinetics applied to substrate oxidation in a chemostat. The Michaelis-Menten parameter, V_{max}, is a relative rather than a specific value. The apparent maximum rate of the enzyme is attained when all of the enzyme sites are saturated with substrate. Therefore, if more enzyme is present at a given substrate concentration, the apparent V_{max} is greater. When the influent ferrous iron concentration is increased, a larger concentration of organic carbon (as a function of ambient substrate concentration) is produced by AK1 (Figure 13).

We can assume that cellular carbon represents approximately 50% of the cell dry weight of AK1 under iron-limited steady state conditions. An increase in cell dry weight (biomass) should result in an increased concentration of ferro-oxidase per unit volume. Therefore, the total activity of the enzyme is greater at increasing influent ferrous iron concentrations as long as the cells remain iron-limited.

The gross iron oxidation rate can be converted to a specific rate. This is accomplished by defining iron oxidation per unit carbon (v_{carbon}) or per cell numbers (v_{cell}). These specific iron oxidation rates are analogous to the specific growth rate constant (μ) described in the Monod growth model. The net iron oxidation rate (v_{carbon}) as a function of threshold corrected ferrous iron concentration is independent of S_R for AK1 (Figure 14). Therefore, enzyme concentration-- not ferric inhibition of ferrous iron oxidation-- appears to be responsible for the results observed in Figures 11 and 12. Additionally, reduced arsenic added to the feed does not affect the net iron oxidation rate of this organism.

The net steady state iron oxidation rate as a function of $S-S_t$ (Figure 14) for AK1 appears to be a linear relationship. Since a theoretical maximum net iron oxidation must exist, the function must approach asymptotically a maximal value. This indicates that the observed rates are significantly lower than the maximum rate obtained by AK1. The maximum rate would presumably occur when much higher ferrous iron concentrations are added to iron-starved batch cultures (see Lang and Brown, 1981). The best-fit line calculated by Model II regression for this relationship did not go through the origin. This may be a result of experimental error (see page 64). It is also possible that the threshold concentration calculated for growth kinetics may not be equal to the threshold concentration for iron oxidation kinetics. For example, the threshold component of

maintenance energy results in the oxidation of ferrous iron but does not result in an increase in biomass. Nevertheless, it is clear from Figure 14 that the influent ferrous iron concentration (from 9.0 to 23.3 mM) does not affect the net iron oxidation kinetics of the AK1 isolate.

Arsenic

AK1 was isolated from placer gold mine drainage containing dissolved arsenic. Speciation of the dissolved arsenic in this stream indicated that arsenite and arsenate species were present in about equal amounts (Brown et al., 1982). Arsenite was present despite the fact that the stream was saturated with oxygen.

In the laboratory, AK1 was tolerant to at least 900 $mg \cdot l^{-1}$ arsenite and arsenate, respectively, when the organism was grown in medium containing ferrous iron (Luong and Brown, 1981).

T. ferrooxidans has been shown to oxidize many reduced metals including cuprous copper (Nielson and Beck, 1972), elemental selenium (Torma and Habashi, 1972), and uranous sulfate (DiSpirito and Tuovinen, 1981; DiSpirito and Tuovinen, 1982a; DiSpirito and Tuovinen, 1982b). Additionally, there is evidence that T. ferrooxidans may be able to obtain energy from some of these inorganic oxidations (DiSpirito and Tuovinen, 1982a; DiSpirito and Tuovinen, 1982b). A mechanism has been proposed (Figure 1) for the generation of energy, reducing power and cellular carbon during iron

oxidation by T. ferrooxidans. This same mechanism could theoretically be used by the acidophile, T. ferrooxidans, to generate energy from thermodynamically favorable reactions involving other reduced inorganic compounds.

For these reasons it was hypothesized that T. ferrooxidans, isolate AK1, might be able to first, oxidize reduced arsenic and second, derive energy from such an oxidation. However, the results of steady state experiments to test this hypothesis were inconclusive. When arsenite was added to the ferrous iron-containing continuous culture medium, no oxidation of the arsenite ion was observed (see Table 3). Additionally, the presence of either 1.33 or 2.67 mM arsenite in the medium did not result in an increase in biomass nor did it appear to have any observable affect on growth kinetic or iron oxidation kinetic parameters which were measured. If arsenite can be used for energy production by isolate AK1, an observable increase in biomass would be expected to occur. These results indicate that, if AK1 is able to use arsenite for energy production, this oxidation is insignificant when ferrous iron is present.

Summary

The importance of the iron-oxidizing thiobacilli in catalyzing biogeochemical cycles has only recently been recognized. With the depletion of high-grade ores, methods of bioleaching for the

recovery of valuable metals are becoming increasingly important. It seems probable that at some time in the future, genetic manipulation may be used to produce highly efficient leaching microorganisms. The iron-limited growth kinetics have not been well described for T. ferrooxidans. The kinetic work that has been published shows inconsistencies in results. These inconsistencies probably result from apparent strain to strain differences, and from the difficulties of culturing this organism, especially in iron-limited continuous cultures.

I have described the growth kinetics, ferrous iron oxidation kinetics and arsenite oxidation potential of an arsenic-tolerant T. ferrooxidans isolate, AK1. The growth of this organism is described in both transient (batch culture) and steady state (continuous culture). In addition, a method has been described for the successful continuous cultivation of T. ferrooxidans in iron-limited continuous cultures. This continuous culture method can be used to more completely describe the growth and iron oxidation kinetics of other strains of T. ferrooxidans. These methods may also be used to investigate the ability of T. ferrooxidans to oxidize and produce energy from reduced metals other than iron. These studies are important for understanding the biogeochemical cycling of many metals, and for application of these microorganisms to biohydrometallurgy.

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